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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Continued progress has been made toward each of the 3 Specific Aims (SAs) of our research project, "Caffeine, adenosine receptors and estrogen in toxin models of Parkinson's disease (PD)". The overarching hypothesis of the project is that multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in PD. To that end we are characterizing the interplay between several environmental agents (pesticides, caffeine and estrogen) that are leading candidate modulators of PD risk. SA#1 (Exp's #2-3) -- Using a series of conditional knockout (KO) mice lacking A2A receptors either in forebrain neurons (CamKIIα-cre/loxP mice) or in astrocytes (CamKIIα-cre/loxP mice), we have demonstrated that the neuroprotective actions caffeine in this PD model depend at least partially on A2ARs other than those located on forebrain (striatal and cortical) neurons and on astrocytes. These results were reported in 2 meeting abstracts in 2006]. SA#2 (Exp's #5) We have validated a powerful virus-based gene delivery system to conditionally knockout adenosine receptors from specific brain regions, enabling us to determine in which brain region(s) adenosine receptors contribute to neurotoxicity in models of PD). The viral (AAV) delivery to the striatum of 'floxed' A2A mice of the cre gene has been found to dose-(titer-)dependently and sequentially produce robust nuclear CRE expression and then A2A receptor disappearance. These results were reported in 2 meeting abstracts in 2006. SA#3 (Exp's #6-7) Our demonstration that estrogen can prevent the neuroprotective effect of caffeine in the mouse MPTP model of PD (SA #3) was fully published in 2006. We reported evidence that endogenous estrogen (in females) and exogenous estrogen (in males and in ovariectomized females) can prevent the protective effect of caffeine on MPTP-induced loss of brain dopamine. Estrogen did not alter caffeine. These findings establish an animal model of estrogen-caffeine interactions in the modification of PD risk in humans, along with the opportunity to understand its molecular mechanisms and develop novel therapeutic strategies for preventing or treating PD

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Introduction

Identifying the mechanisms by which caffeine and more specific A_{2A} antagonists protect dopaminergic neurons in multiple toxin models of Parkinson's disease (PD) will advance our knowledge of the pathophysiology, epidemiology and therapeutics of PD.

The *overarching hypothesis* pursued by this proposal is that **multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease**. Here we endeavor to characterize the interplay between several environmental agents (pesticides, caffeine and estrogen) that are leading candidate modulators of PD risk.
We are pursuing 3 *specific hypotheses:*

1) Caffeine acts through blockade of brain A_{2A}Rs to protect dopaminergic neurons in both acute and chronic toxin models of PD. (***Specific Aim #1***)

2) Caffeine perfusion and focal A_{2A}R inactivation within the striatum are sufficient to attenuate MPTP toxicity, by reducing toxin-induced release of glutamate and/or GABA. (***Specific Aim #2***)

3) Estrogen attenuates the protective effect of caffeine but not the protective of A_{2A}R deletion because it acts by altering caffeine metabolism or A_{2A}R expression. (***Specific Aim #3***)

Statement of Relevance (from our proposal)

A. Parkinson's Disease -

Basic neuroscience significance - The results will improve our understanding of adenosine receptor neurobiology, and will provide insight into the role of endogenous adenosine in basal ganglia biology physiology and PD pathophysiology.

Epidemiological significance - Establishing the ability of caffeine to protect dopaminergic neurons in PD models and identifying a plausible mechanism of action greatly strengthens the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with risk of PD.

Therapeutic significance - With several specific adenosine A_{2A} antagonists emerging as promising therapeutic candidates based on their motor-enhancing (symptom-relieving) action, the prospects for additional neuroprotective benefit substantiated by this project may considerably enhance their therapeutic potential. In addition, identifying a biological basis for caffeine-estrogen interaction in modifying PD risk could also affect recommendations for estrogen replacement strategies in women with PD taking A_{2A} antagonists or caffeine (and *vice versa*). Furthermore, based on evidence that A_{2A}Rs contribute to the neurotoxicity affecting cortical and striatal neurons (as well as dopaminergic neurons), our findings may support novel A_{2A}R-based neuroprotective treatments for a wider range of neurological diseases from stroke to amyotrophic lateral sclerosis (ALS) to Alzheimer's disease.

B. Environmental Neurotoxin Exposure in Military Service – By characterizing the neuroprotective effects of caffeine in a chronic pesticide model of PD (as well as the acute MPTP model), the proposed work will define a prototypical interaction between environmental toxins and protectants in determining the extent of a well-characterized neurological lesion (dopaminergic neuron death). Although there has been no compelling evidence to suggest that the incidence of PD will itself increase in association with military service or combat theatre exposures,[1] putative toxin exposure in the military may be linked to the development of another debilitating neurodegenerative disorder, ALS.[2] Moreover, some objective biological measures in veterans diagnosed with a "Persian Gulf War syndrome" have indicated dysfunction of dopaminergic neurotransmission in the basal ganglia,[3] raising the possibility (together with other data[4]) of altered risk for PD in this group. In any event, establishing a biological precedent for neurotoxin-neuroprotectant interplay in the relatively common disorder of PD, may provide a 'roadmap' that can be used should any neurological illness be confirmed to develop in association with prior military exposures.

C. Understanding the Non-stimulant CNS Effects of Caffeine. The psychoactive agent caffeine has been endorsed for military use at relatively high doses to help maintain operational readiness.[5] This recommendation has been based on a large body of evidence demonstrating sustainment of mental task performance by caffeine, and a lack of evidence for substantial harm at these doses. However, adopting the use of any CNS-active drug by protocol warrants careful consideration of newly appreciated neuronal actions of the agent. Accordingly, the proposed investigation of the novel neuroprotective effect of caffeine and its underlying mechanisms (e.g., altered neurotransmitter release) would be of significance for military programs that provide specific doses of caffeine to personnel to enhance cognitive function.

D. Gender Differences in How Environmental Factors Impact Toxin Susceptibility.
Our investigation of how caffeine and estrogen exposures interact to modify neurotoxin susceptibility in laboratory models of PD may have substantial significance for the human epidemiology that prompted our pursuit of this line of research. In addition, the proposed studies may provide a prototype for modeling how gender and estrogen status interact with environmental exposures of relevance to the military (i.e., neurotoxins, caffeine). A better appreciation of how gender alters susceptibility to environmental toxins or protectants may ultimately lead to a better understanding (and modification) of the differential risks faced by women and men serving in the same military operations.

Body of the Report

Progress during Year 2 on Specific Aims and experiments as laid out in our Statement of Work (SOW [in blue]) is described here in detail.

STATEMENT OF WORK

Specific Aim #1 – to definitively determine whether brain A_{2A}Rs or A_1Rs contribute to dopaminergic neuron degeneration in acute and chronic toxin models of PD, and whether the brain A_{2A}R is required for caffeine's protective effect in these PD models.

[Please see abstract publications in Appendices C and E.]

Hypothesis 1: **Caffeine acts through blockade of brain A_{2A} (not A_1) receptors to protect dopaminergic neurons in both acute (MPTP) and chronic (paraquat/maneb) toxin models of PD.**

Exp# 1 – **Effect of the A_1/A_{2A} receptor double KO in MPTP and paraquat/maneb (Pq/Mb) models**

As reported in our Year 1 progress report we have generated an A_1-A_{2A} double KO line of mice in collaboration with Drs. Jiang-Fan Chen (Boston) and Bertil Fredholm (Sweden). The expansion of this line through double heterozygote ([$A_1^{+/-}$, $A_{2A}^{+/-}$] x [$A_1^{+/-}$, $A_{2A}^{+/-}$]) matings has been slow yielding adequate numbers of well-matched set homozygous A_1 KO [$A_1^{-/-}$, $A_{2A}^{+/+}$], A_{2A} KO [$A_1^{+/+}$, $A_{2A}^{-/-}$], double A_1-A_{2A} KO [$A_1^{-/-}$, $A_{2A}^{-/-}$] and control WT [$A_1^{+/+}$, $A_{2A}^{+/+}$] littermates. (Note that mice of these 4 genotypes comprise only a quarter of all offspring.) The generation of A_1 KO [$A_1^{-/-}$, $A_{2A}^{+/+}$] (i.e., 1/16 of the expected offspring assuming a Mendelian distribution) has been lower than the other 3 genotypes, suggesting a reduced embryonic or perinatal viability in the absence of the A_1 receptor in this mouse strain (C57Bl/6). To simplify the breeding and improve the yield we may cross only A_1 heterozygotes to assess the role of the just A_1 receptor in a neurotoxin model of PD (separately from that of the A_{2A} receptor, which we previously established).

Exp# 2 – **Effect of brain-specific A_{2A} KO in MPTP and Pq/Mb models**.

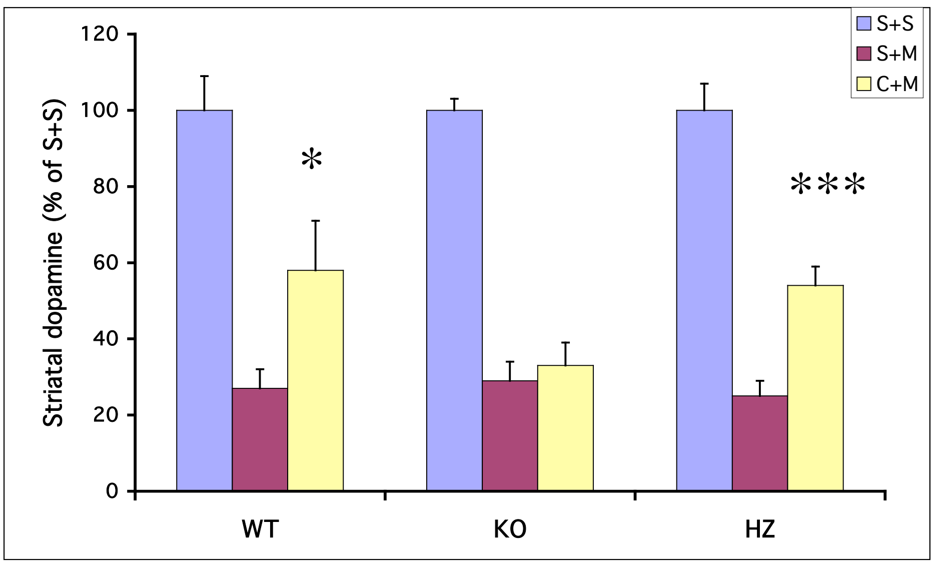
As reported for Year 1, we completed the generation and initial characterization of a conditional (Cre/*loxP* system) KO of post-natal forebrain neuronal A_{2A} receptors. The *CamKIIα* promoter was used to drive expression of the *cre* recombinase gene in postnatal forebrain neurons, and thus to cause selective depletion of striatal neuron A_{2A} receptors following brain development. We recently published (Bastia et al, 2005[6]) evidence of successful forebrain-specific recombination by genetic, autoradiographic and behavioral assessments.

In preliminary experimentes, we found no consistent phenotype of the forebrain neuron A_{2A} receptor (conditional) KO on its own with respect to MPTP toxicity (see below). This allowed us to ask whether these conditional 'brain-specific' A_{2A} (conditional) KO show a reduction in caffeine's neuroprotective effect in this PD model. (See Exp #3 below.)

Exp# 3 – **Brain A_{2A}R-dependence of caffeine's neuroprotective effect**.

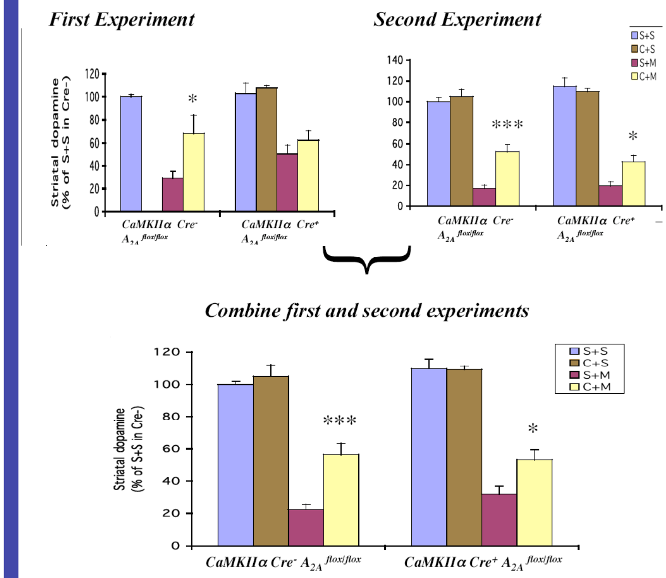
Leading up to this experiment we found that the complete loss of neuroprotection by caffeine in global A_{2A}R KO mice establishes the adenosine A_{2A}R as a critical mediator of caffeine's neuroprotective effects in this model of Parkinson's disease. See Fig. 1. By contrast, caffeine maintains the ability to at least partially protect against MPTP toxicity in conditional KOs of forebrain neuron A_{2A}Rs (Fig. 2) and of astrocyte (Fig. 3) A_{2A}Rs. Together the findings suggest that the neuroprotective actions caffeine in this PD model depend at least partially on A_{2A}Rs other than those located on forebrain (striatal and cortical) neurons and on astrocytes.

Fig. 1. **Caffeine attenuated MPTP-induced striatal dopamine loss in WT and HZ, but not global A_{2A}R KO male mice.**



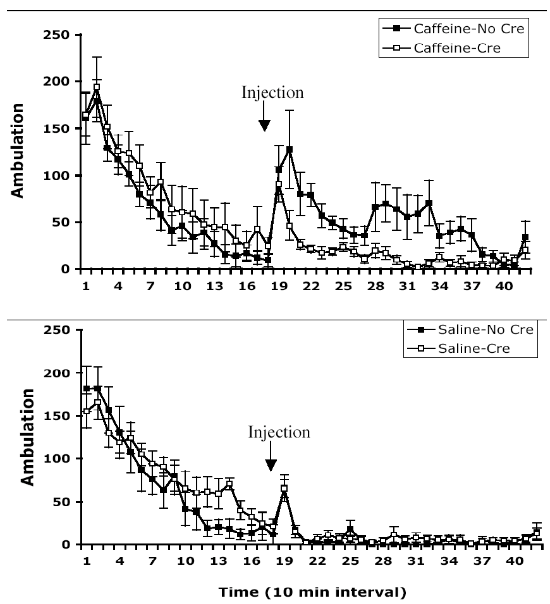
N=3 for saline treatments and n=6-9 for MPTP treatments. *S*, saline; *M*, MPTP (35 mg/kg ip single injection); *C*, Caffeine (25 mg/kg ip 10min before MPTP). *p<0.05, ***p<0.001 compared with respective *S+M* group.

Fig 2. ***A.*** **Caffeine's attenuation of MPTP-induced striatal dopamine loss is at least partially independent of <u>forebrain neuronal</u> A_{2A}Rs.**



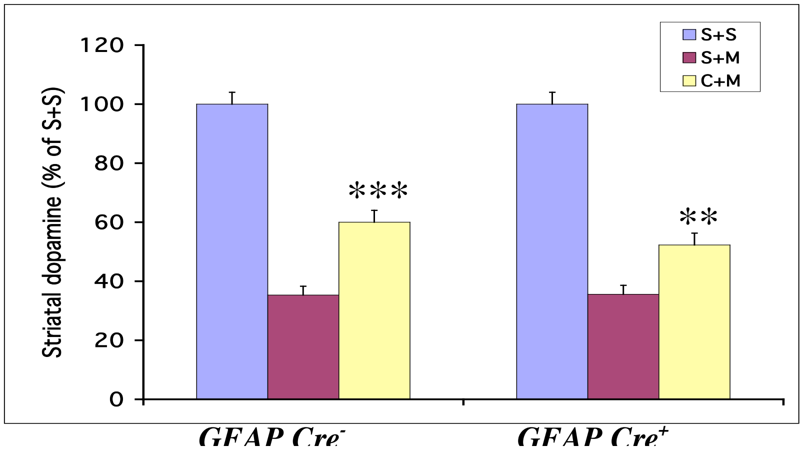
N=3-4 for saline treatments and n=3-13 for MPTP treatments in either first or second experiment. *p<0.05, ***p<0.001 compared with respective *S+M* group.

B. **Locomotion after *caffeine* injection is significantly reduced in <u>forebrain neuron</u> A_{2A}R cKO (*CaMKIIα Cre⁺ A_{2A} flox/flox*) than that in control (*CaMKIIα Cre⁻ A_{2A} flox/flox*) male mice, while locomotions after *saline* injections are similar in A_{2A}R cKO and control mice.**



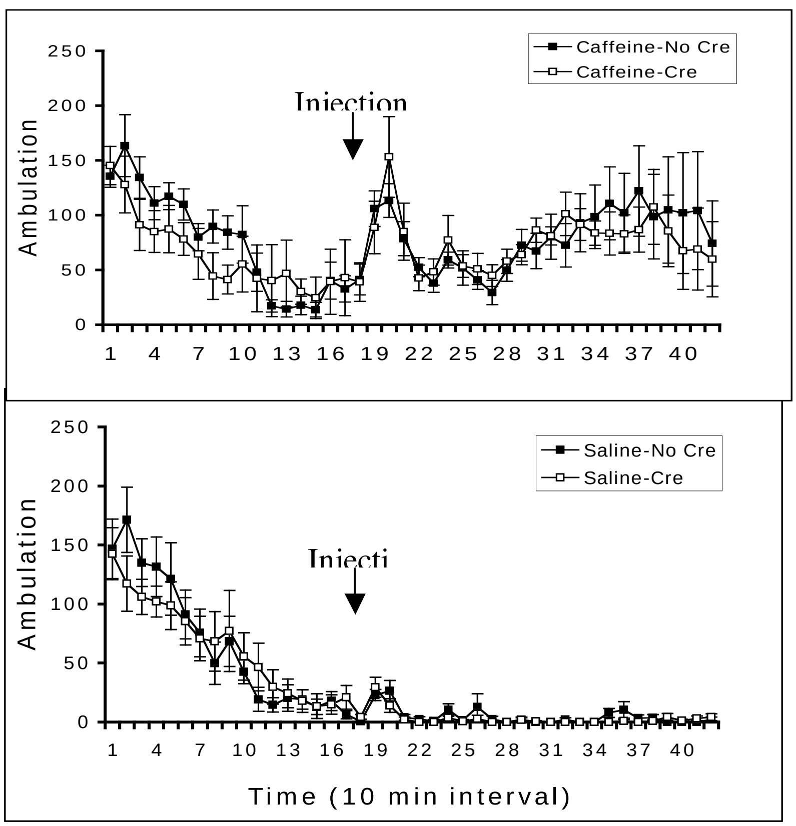
N=8 for each group. The dose of caffeine is 25 mg/kg.

Fig. 3A. **Caffeine attenuated MPTP-induced striatal dopamine loss in control (*GFAP Cre*$^{-}$ *$A_{2A}^{flox/flox}$*) and <u>astrocyte-directed</u> A_{2A}R cKO (*GFAP Cre*$^{+}$ *$A_{2A}^{flox/flox}$*) mice.**



N=6-7 for saline treatments and n=22-39 for MPTP treatments. **p<0.01, ***p<0.001 compared with respective *S+M* group.

3B. **Locomotions after *caffeine* or *saline* injections are similar in control (*GFAP Cre*$^{-}$ *$A_{2A}^{flox/flox}$*) and <u>astrocyte-directed</u> A_{2A}R cKO (*GFAP Cre*$^{+}$ *$A_{2A}^{flox/flox}$*) mice.**



N=8 for each group. The dose of caffeine is 25 mg/kg.

Methods (for Figs. 1-3)

Animals Global A_{2A}R KO mice were generated using a standard displacement target vector. Postnatal forebrain neuron- and astrocyte-directed A_{2A} cKO mice were generated using the Cre-*loxP* system based on the specificities of *CaMKIIα* and *GFAP* gene promoters, respectively. Tissue-specific disruption of the A_{2A}R was confirmed by PCR and western blot. Congenic (N10, C57Bl/6 background) global A_{2A}R KO, wide-type (WT) and heterozygous (HZ) mice, and near congenic (N6, C57Bl/6) $A_{2A}^{flox/flox}$ mice (i.e., homozygous for the floxed allele of the A_{2A}R gene) with or without a *cre* transgene were used for this study. A *cre* transgene was introduced by cross-breeding to either a *CaMKIIα-cre* or *GFAP-cre* lines.

MPTP-induced dopaminergic neurotoxicity. Caffeine (25 mg/kg) or saline were injected intraperitoneally (ip) 10 minutes before MPTP-HCl treatment (35 mg/kg ip single injection). One week after MPTP treatment, the striata were dissected and analyzed for dopamine content using standard reverse-phase HPLC with electrochemical detection. Dopamine content was calculated as pmol/mg of tissue and values are presented within the figures as percent change from respective Saline-Saline treated controls.

Caffeine-stimulated locomotion. Mice were habituated in the testing cages and basal spontaneous locomotion was recorded for at least 180 min. Then locomotor behavior was monitored for another 240 min after caffeine (25 mg/kg ip) or saline injection. Locomotion (*Ambulation*) was scored as the number of adjacent photobeam breaks.

Statistical analyses. The data from striatal dopamine content as well as caffeine-stimulated locomotion were analyzed by two-way ANOVA. Post-hoc comparisons were performed using Bonferroni test. Data values in the figures represent group mean ± SEM.

Specific Aim #2 – to localize the region within brain through which caffeine or A_{2A} receptor inactivation produces its neuroprotective effect in the MPTP model of PD. **(~576 mice)**

[Please see abstract publications in Appendices D and F.]

Hypothesis 2: **Caffeine perfusion and focal A_{2A} receptors inactivation within striatum (but not frontal cortex) are sufficient to attenuate MPTP toxicity, by reducing toxin-induced striatal release of glutamate and/or GABA.**

Exp# 4 – **Effect of intracerebral caffeine perfusion on MPTP-induced neuro-transmitter overflow and toxicity**:

In preparation for this experiment with local administration of caffeine, we are continuing to characterize (using microdialysis) MPTP-induced neurotransmitter overflow in the striatum and its modulation by systemic caffeine.

Exp# 5 – **Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed A_{2A}R mice**: Homozygous floxed A_{2A}R mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP. One week later infusion needle track will be localized histochemically, while dopaminergic neuron integrity will be visualized by striatal DAT binding and nigral TH-IR counts will be assessed as in Exp #4.

During Year 2 we have made further progress toward this experiment in characterizing the viral Cre/*loxP* conditional KO method that was adopted in Year 1. We have demonstrated that the resulting unilateral local recombination and disruption of the A_{2A} gene, and in turn the elimination of striatal A_{2A} receptors are dose-(titer-)dependent and occur sequentially. Working with several serotypes of AAV-*cre* and AAV-*GFP* (provided through a collaboration with Dr. Miguel Esteves), Dr. Augusta Pisanu has now demonstrated that:

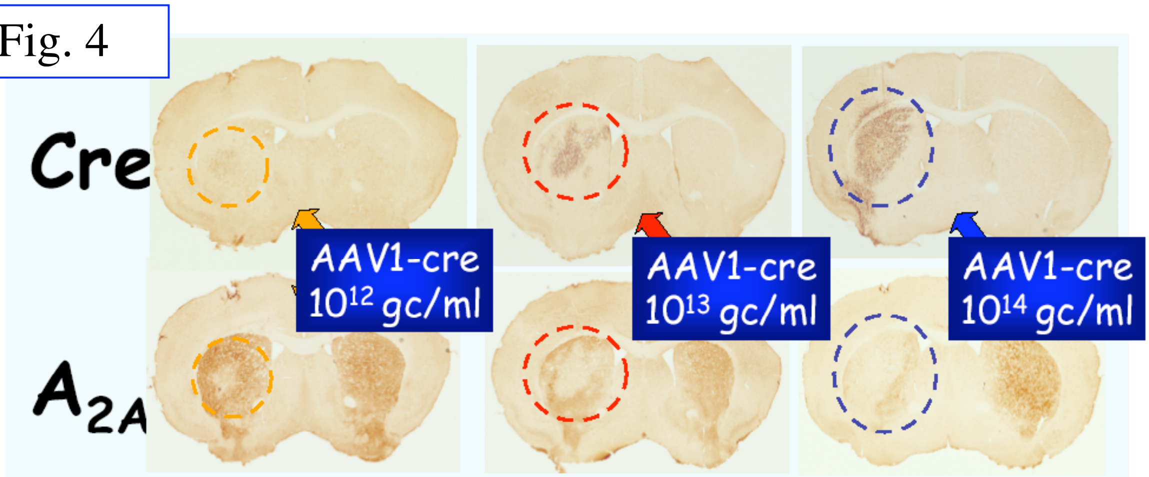
- Using this AAV-Cre/*lox*P system we were able to obtain a conditional KO of the A_{2A}R in the striatum of adult mice. Whereas the Cre expression was detectable within 8 days after injection (Fig. 5B), the A_{2A}R-IR loss was not evident until the 16th day (Fig. 5D), presumably due to the stability of the already expressed receptors, and reached a maximum level by the 32nd day post injection. (Figs. 4 and 5D.)

- This viral *cre*-based Cre/*lox*P conditional KO of the A_{2A}R was also a titer-dependent phenomenon since an increasing viral titer produces a greater the number of infected cells and further reductions in A_{2A}R-IR. (Figs. 4 and 5A and C.)

- The viral vector titer not only determines the intensity of $A_{2A}R$ KO, but also the spatial extent of the infection, and consequently the $A_{2A}R$ loss (Fig. 4), suggesting that the virus particles may diffuse through the brain parenchyma until they find a "free" binding site that stops their diffusion and let them into a host cell. Is it important to consider whether an excessive viral concentration could produce an over-expression of Cre, which is capable of inducing cell toxicity (as detected in the center of the infected area in some mice infected with the highest AAV-cre titer, data not shown).

- In this model, the AAV-Cre/*loxP* conditional KO system provides a temporally and spatially controlled method to precisely eliminate $A_{2A}R$ in discrete brain regions providing additional advantages in exploring the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

Fig. 4



This methodological advance will allow us to dissect A_{2A} receptor involvement in neurotoxin models of PD with an unprecedented combination of anatomical and molecular precision. We are now initiating the MPTP studies in these mice lacking A_{2A} receptors in discrete brain regions.

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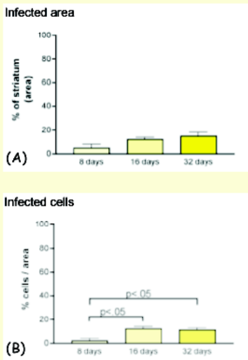
Fig. 5

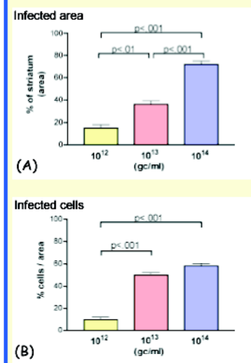
A Time-course of Cre expression



The extent of AAV-cre infection (infected area) is expressed as percentage of the striatal area (A). Cre positive cells are detectable by 8 days post injection and no statistical difference was found in the extent of AAV-cre diffusion 8, 16 or 32 days after viral injection, even though the percentage of Cre-IR cell bodies in the infected area (B) is still very low 8 days post infusion and reached a stable level on the 16th day.

B Dose-dependence of Cre expression

<u>Cre-IR 32 days after infusion of 1 μl of 10^{12}, 10^{13} or 10^{14} gc/ml AAV1-cre solution</u>

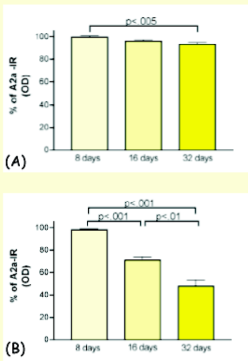


The extent of AAV-cre infection (infected area), expressed as percentage of the striatal area, depends on the titer of AAV-cre infused (A): increasing the AAV-cre titer 10 times doubles the extent of the infected area.

The percentage of Cre-IR cells in the infected area is shown in panel B

C Time-course of A_{2A} loss

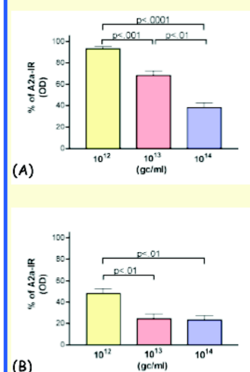
<u>A_{2A}-IR after infusion of 1 μl of AAV1-cre (10^{12} gc/ml) analyzed at 3 different time points.</u>



The extent of $A_{2A}R$ loss is expressed as reduction of $A_{2A}R$-IR optical density (OD) in the infected striatum, compared to the intact one (A). The lower panel shows the level of residual $A_{2A}R$-IR in the infected area (defined by Cre-IR localization in each adjacent section) expressed as reduction of OD compared to background. $A_{2A}R$-IR reduction was almost undetectable 8 days post injection and reached the higher level 32 days post injection.

D Dose-dependence of A_{2A} loss

<u>A_{2A}-IR 32 days after infusion of 1 μl of 10^{12}, 10^{13} or 10^{14} gc/ml AAV1-cre solution</u>



In the upper panel is shown the titer-dependence of the $A_{2A}R$-IR reduction in the infected striatum calculated as the percentage of the $A_{2A}R$-IR OD within the striatum compared to that within the untreated striatum.

The lower panel shows the extent of $A_{2A}R$ KO reached in the infected area (defined by Cre-IR localization in each adjacent section) expressed as a reduction of $A_{2A}R$-IR OD compared to the equivalent region of the intact side.

Methods for Figs. 4 and 5

Mice with a *floxed* $A_{2A}R$ gene were generated by insertion of *lox*P sequences within the introns flanking the exon 2 of the $A_{2A}R$ gene (YJ Day and J linden, unpublished results). Homozygous adult male mice were anesthetized with an i.p. injection of Avertin and positioned in a stereotaxic frame for injection into the left striatum (AP: +0.7; ML: +2; DV: -2.8) of 1 μl of AAV1-cre (9.3x10^{13}, 1x10^{13} or 1x10^{12} gc/ml) at a rate of 0.1 μl/min by using a 30 gauge needle connected to a 50μl Hamilton syringe driven by a microinfusion pump.

After 8, 16 or 32 days mice were anesthetized and intracardially perfused 10 ml of ice-cold saline followed by 50 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer. After perfusion brains were removed and incubated overnight in the same fixative than cryoprotected by incubation in phosphate buffered 30% sucrose. Serial 25 μm-thick coronal sections were cut on a freezing microtome and collected in 50 mM Tris buffer. Adjacent sections starting from the rostral part of the striatum were collected for immunohistochemical staining for Cre recombinase (1:2000 Novagen anti-Cre rabbit polyclonal antibody) and $A_{2A}R$ (1:200 Santa Cruz anti A2AR goat polyclonal antibody). Donkey anti-species antibodies conjugated to biotin, Vectastain ABC Kit and fast DAB Kit were used for detecting primary antibodies. Immunostainig controls were done without the primary antibody. Quantification of Cre and A2aR-immunoreactivity (IR) was performed every sixth section at 10X and 40X magnification. Statistical analysis was performed by Oneway ANOVA followed by a Tukey post hoc analysis of means difference between groups.

Specific Aim #3 – to investigate caffeine-estrogen interactions in the MPTP model of PD by determining the effect estrogen replacement on the neuroprotective phenotype of A_{2A} KO mice, and exploring potential peripheral and CNS mechanisms contributing to caffeine's reduced neuroprotective efficacy in the presence of estrogen.

[Please see manuscript publications in Appendix A.]

With support from this award we have now published our studies of estrogen-caffeine interaction in the MPTP model of Parkinson's disease, as detailed in the attached final manuscript (Appendix A). Our results demonstrate that estrogen reduces caffeine's neuroprotective effect against MPTP toxicity in both male and female mice. In the context of human epidemiology on PD, our findings suggest a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

Key Research Accomplishments (in Year 2)

- Confirmation of adenosine A_{2A} receptor requirement for caffeine's neuroprotective effect in the MPTP model of neurodegeneration in Parkinson's disease.
- Demonstration that these receptors on forebrain neurons or astrocytes do not fully account for caffeine's neuroprotective action against MPTP toxicity, suggesting surprisingly that other neurons or glial cells are the source of the A_{2A} receptors through which caffeine is acting.
- Characterization of a powerful Cre-*loxP* conditional knockout methodology that will allow us to dissect -- with exceptional molecular and anatomical precision -- the role of the adenosine A_{2A} receptor in caffeine's influence on dopaminergic neuron injury in neurotoxin models of PD
- Publication of our systematic demonstration that estrogen can prevent caffeine's neuroprotective effect against dopaminergic neuron injury in the MPTP mosue model of Parkinson's disease (PD). In so doing, we have provided a biological basis for the consistent epidemiological finding that caffeine is associated with a reduced risk of PD in men and in women who have not had estrogen replacement therapy, but not in women who have had estrogen replacement therapy.
- Publications (with acknowledgements citing W81XWH-04-1-0881/ USAMRAA)
    a. Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen reduces the neuroprotective effect of caffeine in a mouse model of Parkinson's disease. Program No. 665.12. 2005 Washington, DC: Society for Neuroscience. [Abstract]
    b. Hauser R, Schwarzschild MA. 2005. Adenosine A_{2A} receptors for Parkinson's disease. *Drugs and Aging* 22:471-482. [Review]
    c. Xu K, Xu Y, Brown-Jermyn D, Chen J-F, Ascherio A, Dluzen D, Schwarzschild MA. Estrogen prevents neuroprotection by caffeine in the mouse MPTP model of Parkinson's disease. [submitted to *J Neurosci*; status: acceptable pending revision]

Reportable Outcomes

1) Publications (with acknowledgements citing W81XWH-04-1-0881/ USAMRAA)

- Xu K, Xu Y, Brown-Jermyn D, Chen JF, Ascherio A, Dluzen DE, Schwarzschild MA. (2006) Estrogen prevents neuroprotection by caffeine in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *J Neurosci*. 26:535-541. (See Appendix A.)
- Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M. (2006) Targeting adenosine A_{2A} receptors in Parkinson's disease. *Trends Neurosci*. 29:647-654. [Review] (See Appendix B.)
- Xu K, Xu Y-H, Chen J-F, Schwarzschild MA. (2006) The neuroprotection by caffeine in the MPTP model of Parkinson's disease is lost in adenosine A_{2A}

knockout mice. *Targeting Adenosine A_{2A} Receptors in Parkinson's Disease and Other CNS Disorders*. Boston May 17-19, 2006 www.A2APD.org P30 [abstract]. (See Appendix C.)

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2) Major presentations (with acknowledgements including DoD/USAMRAA/NETRP)

- January 4, 2006 – (Piscatway) University of New Jersey Medicine & Dentistry – grand rounds, "*Caffeine, adenosine A_{2A} receptors and Parkinson's disease*"
- February 25, 2006 –World Parkinson Congress – Washington, DC "*Neuroprotective and Anti-dyskinetic Potential of Adenosine A_{2A} Blockers*"
- March 27, 2006 –American Chemical Society – Atlanta [Co-chair] Symposium: *Current and Future Potential Drug Therapy for Parkinson's Disease*; "*Progress on the etiology, modeling and treatment of Parkinson's disease*"
- May 18, 2006 – *Targeting Adenosine A_{2A} Receptors in Parkinson's Disease and Other CNS Disorders*. (Organizer/Co-chair) Boston May 17-19, "*A_{2A} in L-dopa sensitization/dyskinesia models*".
- October 18, 2006 – Society for Neuroscience – Atlanta (Symposium: Purinergic Signaling in Neuron-Glia Interactions) "*Adenosine A_{2A} Receptors in Neurodegeneration*" {Eweson Lectureship}

Conclusions

Central hypothesis: Multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease. Our initial progress under this award supports the central hypothesis, particularly with respect to caffeine-estrogen interactions in models of PD (**SA 3**). Critical to our ability to successfully pursue the Specific Aims of our research program, we have made substantial progress in establishing and charactering the key KO methodologies of this project. We expect to build on our conceptual and technical advances of the first two years in pursuing the key experiments of the project as originally proposed in our SOW.

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Appendix A

Neurobiology of Disease

Estrogen Prevents Neuroprotection by Caffeine in the Mouse 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Model of Parkinson's Disease

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Epidemiological studies have strongly linked caffeine consumption with a reduced risk of developing Parkinson's disease (PD) in men. Interestingly, in women, this inverse association is present only in those who have not taken postmenopausal estrogens, suggesting an interaction between the influences of estrogen and caffeine use on the risk of PD. To explore a possible biological basis for this interaction, we systematically investigated how the neuroprotective effect of caffeine is influenced by gender, ovariectomy (OVX), and then exogenous estrogen in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD. (1) Caffeine treatment produced a dose-dependent attenuation of MPTP-induced striatal dopamine loss in both young and retired breeder (RB) male, but not female, mice. (2) In female mice (both young and RB), caffeine was less potent or altogether ineffective as a neuroprotectant after sham surgery compared to OVX or after OVX plus estrogen replacement compared to OVX plus placebo treatment. (3) Estrogen treatment also prevented the protection of caffeine against dopamine loss in young male mice. (4) Consistent with the putative protective effect of estrogen, female and OVX plus estrogen mice were relatively resistant to MPTP toxicity compared to male and OVX plus placebo mice, respectively. (5) There was no overall difference in brain levels of caffeine and its metabolites between OVX plus placebo and OVX plus estrogen mice. Together, these results suggest that estrogen can occlude and thereby prevent the neuroprotective effect of caffeine in a model of PD neurodegeneration, supporting a biological basis for the interaction between estrogen and caffeine in modifying the risk of PD.

Key words: **adenosine A_{2A} receptor; dopamine; gender; methylxanthine; ovariectomy; striatum**

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is pathologically well characterized. However, the etiology of PD remains unclear. Twin studies (Tanner et al., 1999; Vieregge et al., 1999; Wirdefeldt et al., 2004) have suggested that nongenetic factors, such as environmental exposures or random cellular events that occur during aging, play a prominent role in promoting the development of typical PD.

A major negative risk factor for PD has been identified recently as the consumption of caffeine. Multiple retrospective as well as several large prospective epidemiological studies have demonstrated that among dietary factors, previous coffee or tea drinking are consistently associated with a reduced risk of developing PD even after accounting for smoking and other potential confounding factors (Benedetti et al., 2000; Ross et al., 2000; Ascherio et al., 2001, 2004). The incidence of PD declines steadily with increasing intake of caffeine or coffee (but not decaffeinated coffee).

The mechanisms that underlie this epidemiological correlation remain unclear. One hypothesis that caffeine might represent a protective environmental factor in PD is supported by our findings that caffeine can protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal neurodegeneration in mice (Chen et al., 2001; Oztas et al., 2002). Moreover, the caffeine metabolites paraxanthine and theophylline provide similar attenuations of MPTP-induced dopaminergic toxicity (Xu et al., 2002b). In contrast to its locomotor stimulant effect, the neuroprotectant effect of caffeine does not show tolerance after chronic caffeine exposure (Xu et al., 2002a). Recently, the protection of caffeine against dopaminergic neuron loss and associated behavior changes was confirmed in the 6-OHDA rat model of PD (Joghataie et al., 2004). Together, the protective effects of caffeine and its metabolites in rodent models of PD support a causal basis for the inverse relationship between

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human caffeine consumption and the risk of subsequently developing PD.

Interestingly, the negative link between caffeine intake and risk of developing PD has been observed consistently in men but not in women (Benedetti et al., 2000; Ascherio et al., 2001, 2004). Overall, there was no clear relationship between PD and caffeine intake in two large prospectively followed populations of women (Ascherio et al., 2001, 2004). When these women were further divided by their estrogen use status after menopause, a negative association between caffeine intake and risk of PD similar to men was observed in those women who had never used estrogen replacement therapy but not in those who had ever used it (Ascherio et al., 2003, 2004). These results suggest that estrogen replacement therapy may prevent the beneficial effect of caffeine in reducing the risk of developing PD.

To address the possibility that estrogen, which can serve as a neuroprotectant in its own right, may interfere with neuroprotection by caffeine against dopaminergic neurotoxicity, we investigated their interaction in the MPTP mouse model of PD. We first assessed the difference in neuroprotection by caffeine in male and female mice. Then we systematically investigated the effect of exogenous estrogen on the neuroprotection of caffeine. Finally, we explored the effect of estrogen on the metabolism of caffeine as a possible mechanism of interaction between estrogen and caffeine.

Materials and Methods

Animals, ovariectomy, and estrogen replacement. Young (~10 weeks old) or retired breeder (6–9 months old) male and female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used in these experiments. All experiments were performed in accordance with Massachusetts General Hospital and National Institutes of Health guidelines on the ethical use of animals. The mice were housed five per cage with *ad libitum* access to food and water and were maintained at a constant temperature and humidity with a 12 h light/dark cycle. To remove the main source of female hormones, bilateral ovariectomies were performed under anesthesia using Avertin (2% 2,2,2-tribromoethanol and 1% amyl alcohol; 20 ml/kg, i.p.) at either Charles River Laboratories or Massachusetts General Hospital. Sham operations were also included, in which all of the other procedures were the same, except for removal of the ovaries. In the experiments in which estrogen replacement was used, placebo or estrogen pellets (17β-estradiol, 0.1 mg per pellet, 21 d release; Innovative Research of America, Sarasota, FL) were implanted at the neck of mice under anesthesia 7–10 d after ovariectomy. This estrogen regimen was used because it produces an approximate replacement of physiological levels within the serum in ovariectomized (OVX) mice (Gao and Dluzen, 2001).

Caffeine and MPTP treatment. Different doses of caffeine (5, 10, 20, or 40 mg/kg) or saline were injected intraperitoneally 10 min before MPTP (40 mg/kg, i.p.) or saline injection ($n = 3$–7 for saline treatments and $n =$ 4–15 for MPTP treatments). The extent of the caffeine dose range used across experiments varied depending on the number of mice available. In experiments in which ovariectomy and/or estrogen pellets were implanted, caffeine and MPTP treatments were performed 10 d after ovariectomy or estrogen implantation, whichever came later, to assure that estrogen levels were depleted or maintained at a constant level, respectively.

Dopamine and 3,4-dihydroxyphenylacetic acid measurement. One week after MPTP treatment, mice were killed by rapid cervical dislocation. The striatum was dissected out from the right cerebral hemisphere, frozen on dry ice, and stored at −80°C until use. Each striatum was weighed and extracted with 150 mM phosphoric acid and 0.2 mM EDTA. The striatum was homogenized and centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatants were analyzed for dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) content using standard reverse-phase HPLC with electrochemical detection (ESA, Chelmsford, MA). Biogenic amines were separated on a C-18 5 μm sphere column (Varian, Palo Alto, CA). The mobile phase consisted of 0.1 M sodium phosphate monobasic, 0.1 mM EDTA, 0.18 mM sodium octyl sulfate, and 8% methanol in filtered distilled water. The final pH of 3.3 was obtained with the addition of concentrated phosphoric acid, and the mobile phase was filtered and degassed before use. The dopamine and DOPAC contents were calculated as picomoles per milligram of tissue, and these values are presented within the figures as percentage of change from respective saline–saline-treated controls.

Measurement of caffeine and its metabolites. Ten days after estrogen or placebo pellet implantation, OVX retired breeder female mice were treated with saline or caffeine (5 or 40 mg/kg, i.p.; $n = 1$ for saline and $n =$ 5 for caffeine). The mice were killed at 10, 30, 60, 120, 180, 240, or 360 min after injection by rapid cervical dislocation. The right cerebral hemisphere was dissected out, frozen on dry ice, and stored at −80°C until use. Each brain tissue was homogenized in 0.1 M monobasic sodium phosphate with a volume 10 times of tissue weight and centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatants were analyzed using liquid chromatography/mass spectrometry for determination of caffeine and its three metabolites, paraxanthine, theophylline, and theobromine. The lower limit of quantitation was 30 ng/ml. The analysis of caffeine and its metabolites was gratefully performed by Drs. R. L. Foltz and D. Andrenyak (Center for Human Toxicology, University of Utah, Salt Lake City, UT).

Statistical analyses. The data from striatal dopamine and DOPAC content as well as caffeine/metabolite measurements were analyzed by two-way ANOVA. *Post hoc* comparisons were performed using Fisher's least significant difference test. Data values in the figures represent the group mean ± SEM.

Results

Gender differences in the attenuation of MPTP toxicity by caffeine

The dose dependence of the neuroprotective effect of caffeine in the MPTP model of PD was first compared between intact male and female mice.

Young mice

MPTP treatment depleted the striatal dopamine level measured 1 week later down to 38% of control (saline treated) in ~10-week-old male mice (Fig. 1*A*). Caffeine pretreatment attenuated MPTP-induced dopamine loss in a dose-dependent manner in these young males, with a maximal effect (of doubling residual dopamine levels) achieved at 10 mg/kg. In female mice of the same age, MPTP depleted striatal dopamine levels to 62% of control levels (in saline-treated females). This reduction is significantly less than that observed in their male counterparts ($p <$ 0.05), agreeing with previous reports (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998) that MPTP induced less dopaminergic toxicity in female than male mice. However, in contrast to male mice, female mice showed no attenuation of MPTP toxicity after lower doses (5, 10, or 20 mg/kg) of caffeine pretreatment. Only the highest dose (40 mg/kg) of caffeine protected against MPTP. Simultaneous measurement of DOPAC (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material), the major metabolite of dopamine in mouse, revealed that lower doses of caffeine pretreatment (10 or 20 mg/kg) significantly attenuated MPTP-induced depletion in male mice, whereas only the highest dose of caffeine pretreatment (40 mg/kg) provided similar protection in female mice.

Retired breeders

We also examined the neuroprotection of caffeine against MPTP toxicity in older (6–9 months old) retired breeder mice (Fig. 1*B*). Because these mice have passed their peak reproductive age, their comparison may be more relevant to gender differences in epidemiological studies of PD, which is usually diagnosed in people

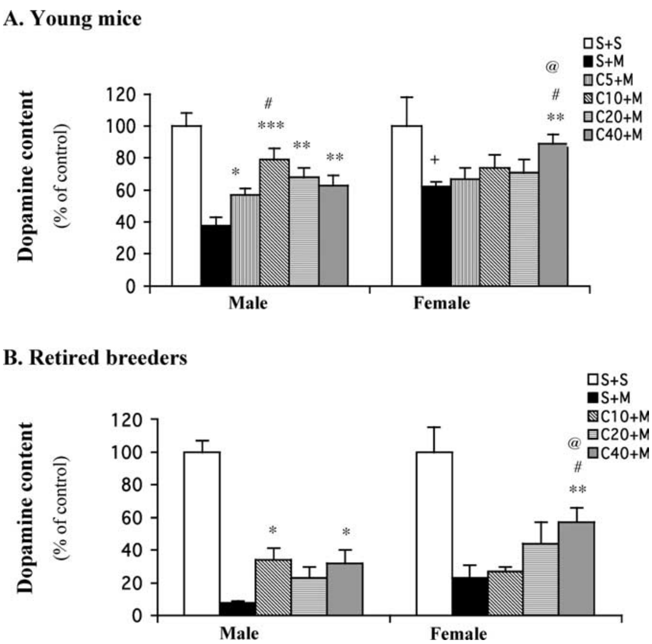

Figure 1. Caffeine dose-dependently attenuates MPTP-induced dopamine depletion in male but not female C57BL/6 mice, either young (~10 weeks old; ***A***) or retired breeder (6–9 months old; ***B***). Caffeine (5–40 mg/kg, i.p.) or saline was administered 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 4$–12) or saline ($n = 3$–6). One week later, striatal dopamine content was determined. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations (in picomoles per milligram of tissue) of these controls are 71.8 ± 2.4 and 70.6 ± 2.6 (***A***) and 80 ± 9 and 92 ± 9.8 (***B***) for males and females, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. ***A***, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.05$ compared with the respective C5+M group; $^{+}p < 0.05$ compared with S+M in male mice; $^{@}p < 0.01$ compared with C40+M in male mice. ***B***, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the respective S+M group; $^{\#}p < 0.05$ compared with the respective C10+M group; $^{@}p < 0.05$ compared with C40+M in male mice. S, Saline; M, MPTP; C5–40, caffeine at 5–40 mg/kg.

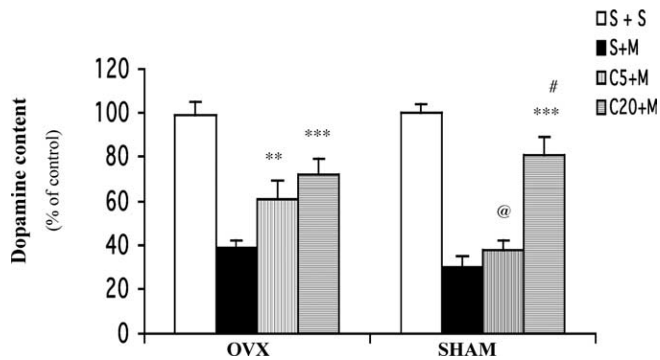

Figure 2. A low dose of caffeine attenuates MPTP-induced dopamine depletion in OVX but not sham-operated young female mice. Ten days after ovariectomy or sham operation, mice received caffeine (5 or 20 mg/kg, i.p.) or saline administration 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 8$) or saline ($n = 5$–7). One week later, striatal dopamine content was determined. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations of these controls are 61 ± 3.7 and 67 ± 2.7 pmol/mg of tissue for OVX and sham mice, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.001$ compared with the respective C5+M group; $^{@}p < 0.01$ compared with C5+M in OVX mice. S, Saline; M, MPTP; C5 or C20, caffeine at 5 or 20 mg/kg.

>50 years old. Not surprisingly, MPTP treatment induced a greater biochemical lesion in older mice for either gender (Fig. 1, compare *A*, *B*), as reported previously (Irwin et al., 1992). In male retired breeders, MPTP treatment depleted striatal dopamine levels to 8% of unlesioned controls (Fig. 1*B*). As in the younger male mice, caffeine pretreatment ≥10 mg/kg again significantly attenuated MPTP-induced dopamine depletion. In female retired breeders, MPTP treatment depleted striatal dopamine levels to 23% of control levels. Again, female retired breeder mice also showed less MPTP toxicity compared with that of male mice. However, just as in young mice, pretreatment with only the highest dose of caffeine (40 mg/kg) significantly attenuated the dopamine loss. Caffeine pretreatment, at all doses tested (10, 20, or 40 mg/kg), also attenuated loss of DOPAC in male mice, whereas only higher doses provided similar protection in female mice (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material).

Ovariectomy increases the potency of the protective effect of caffeine

To investigate whether the gender difference in the neuroprotective effects of caffeine may relate to differences in female hormone status, we examined the effect of caffeine on MPTP toxicity in young female mice whose ovaries, the main site of female hormone production, were removed (OVX) or in sham-operated female littermates (Fig. 2). MPTP treatment depleted striatal dopamine levels in both OVX and sham-operated mice. However, the lower dose of caffeine tested significantly increased residual striatal dopamine levels only in OVX females (Fig. 1, males). In contrast, sham-operated females (like intact females) required pretreatment with a higher dose of caffeine for attenuation of MPTP-induced dopamine loss.

Estrogen attenuates the neuroprotection of caffeine in ovariectomized female mice

Young mice

To determine whether estrogen, the main female sex hormone, can account for the above-identified ovarian influence on neuroprotection by caffeine, we examined the effect of prolonged estrogen replacement on neuroprotection by caffeine in female mice depleted of endogenous estrogen (Fig. 3*A*). These OVX mice were implanted with subcutaneous pellets containing placebo or estrogen (17β-estradiol), which is continuously released to maintain steady-state concentrations for 21 d. MPTP treatment reduced striatal dopamine content to 27% of control in placebo-treated mice (OVX+P). The same MPTP exposure in OVX mice treated with estrogen pellets (OVX+E) depleted striatal dopamine levels, but to a significantly lesser extent (down to 39% of control), consistent with previous reports (Dluzen et al., 1996; Miller et al., 1998). In these OVX+P mice, pretreatment with caffeine, both at 10 and 20 mg/kg, significantly attenuated MPTP-induced striatal dopamine loss. In mice that were receiving estrogen replacement (OVX+E), however, only the higher dose of caffeine (20 mg/kg) provided significant protection. The striatal levels of DOPAC (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material) demonstrated a similar phenomenon.

Retired breeders

To better model estrogen replacement in the menopausal state, a parallel experiment was conducted in retired breeder (rather than young) mice (Fig. 3*B*). Again, estrogen by itself significantly attenuated MPTP-induced striatal dopamine loss. MPTP depleted dopamine levels to 23% of control in OVX+P mice, whereas it reduced them to 36% of control in OVX+E mice. In keeping with

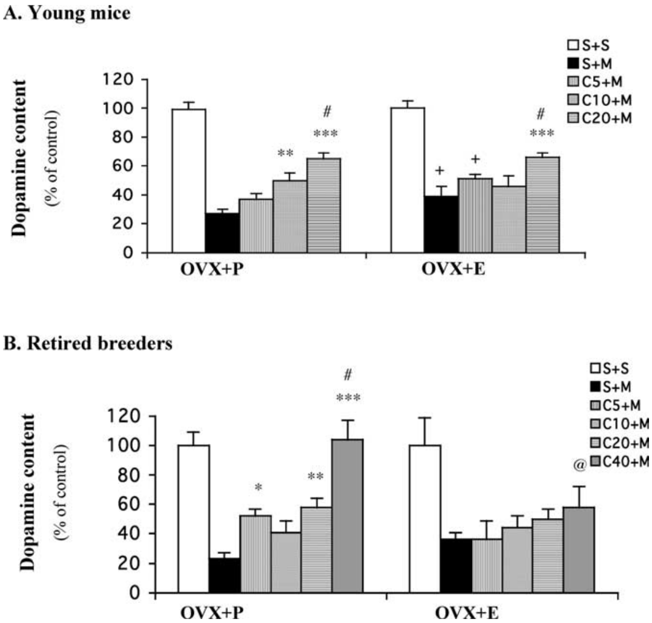

Figure 3. Caffeine attenuates MPTP-induced dopamine depletion in OVX female mice receiving placebo (OVX+P) but not estrogen (OVX+E) replacement (***A***, young mice; ***B***, retired breeders). Ten days after ovariectomy, mice were implanted with placebo or estrogen pellets (17β-estradiol, 0.1 mg per pellet, 21 d release) subcutaneously. Ten days later, caffeine (5–40 mg/kg, i.p.) or saline was administered 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 6$–15) or saline ($n = 3$–6). Striatal dopamine content was determined 1 week after MPTP. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations (in picomoles per milligram of tissue) of these controls are 72 ± 4 and 78 ± 4.7 (***A***) and 78.9 ± 7.3 and 73.9 ± 7.7 (***B***) for males and females, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. ***A***, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.05$ compared with the respective C5+M and C10+M groups; $^{+}p < 0.05$ compared with the respective S+M or C5+M in OVX+P mice. ***B***, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the respective S+M group; $^{\#}p < 0.001$ compared with the respective C5+M, C10+M, or C20+M group; $^{@}p < 0.05$ compared with C40+M in OVX+P mice. S, Saline; M, MPTP; C5–40, caffeine at 5–40 mg/kg.

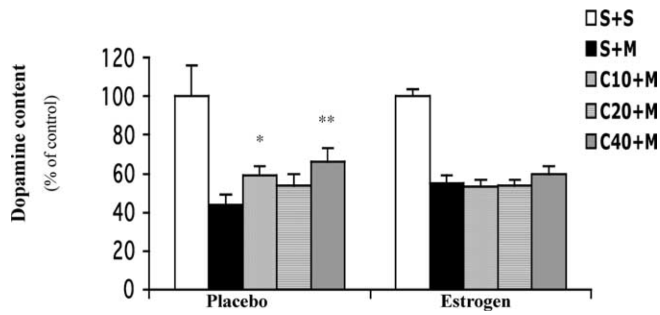

Figure 4. Caffeine attenuates MPTP-induced dopamine depletion in young male mice receiving placebo but not estrogen treatment. Mice were implanted with placebo or estrogen pellets. Ten days later, caffeine (10–40 mg/kg, i.p.) or saline was administered 10 min before a single dose of MPTP (40 mg/kg, i.p.; $n = 8$–9) or saline ($n = 3$). Striatal dopamine content was determined 1 week after MPTP. The bars represent striatal dopamine levels (mean ± SEM) calculated as a percentage of their respective controls (i.e., S+S group). The dopamine concentrations of these controls are 79 ± 10.6 and 80.6 ± 4.2 pmol/mg of tissue for placebo- and estrogen-treated mice, respectively. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the respective S+M group. S, Saline; M, MPTP; C10–40, caffeine at 10–40 mg/kg.

observations in young mice, caffeine pretreatments (5, 20, or 40 mg/kg) significantly attenuated MPTP-induced dopamine loss in the OVX+P retired breeder mice, with complete reversal achieved at the highest dose of caffeine. However, in the setting of estrogen replacement in these OVX retired breeders, caffeine pretreatment did not confer protection at any dose tested. The measurement of DOPAC (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material) yielded a similar set of findings, with caffeine pretreatment (20 or 40 mg/kg) significantly reducing DOPAC loss in OVX+P but not OVX+E mice. Together, these results demonstrate that estrogen replacement can reduce or abolish the neuroprotective effect of caffeine on MPTP toxicity in young and older OVX females.

Estrogen can prevent neuroprotection by caffeine in male mice

The above effects of manipulating endogenous and exogenous estrogen in female mice suggest that estrogen status may be the key factor accounting for the gender difference in the neuroprotective action of caffeine in the MPTP model of PD. To determine whether the neuroprotective effect of caffeine in males indeed relies on the absence or relatively low levels of estrogen, we assessed the effects of caffeine on MPTP toxicity in male mice implanted with placebo or estrogen pellets (Fig. 4). MPTP treatment depleted striatal dopamine levels to 44% and 55% of their respective control level in male mice pretreated for 21 d with placebo or estrogen, suggestive of a mild protective estrogen effect as repeatedly observed above. In the placebo-implanted males, caffeine pretreatments significantly attenuated striatal dopamine loss. However, no dose of caffeine pretreatment protected the male mice treated with estrogen pellets. These results demonstrate that estrogen can abolish the neuroprotection of caffeine not only in female mice but also in male mice.

The effect of estrogen on caffeine metabolism in ovariectomized female mice

As a first step to understanding the mechanism of the influence of estrogen on neuroprotection by caffeine, we measured the brain levels of caffeine (trimethylxanthine) and its major demethylation metabolites (dimethylxanthines; i.e., paraxanthine, theophylline, and theobromine) at multiple time points after treatment with caffeine in OVX retired breeder female mice treated with placebo or estrogen pellets. Because estrogen status most consistently modulated the protective effect of lower doses of caffeine, we assessed the effect of estrogen on the CNS pharmacokinetics of caffeine administered intraperitoneally at 5 mg/kg (Fig. 5*A*). Brain concentrations of caffeine, which were maximal within 10 min of injection and declined with a half-life of ~50 min, were indistinguishable between the estrogen and placebo groups at all time points measured. When caffeine was administered at this low dose, the concentrations of its metabolites were below the limits of reliable detection. There was no measurable caffeine after saline injection.

As demonstrated above (Fig. 3*B*), in these OVX retired breeder mice, caffeine pretreatments attenuated dopamine depletion in placebo-treated but not in estrogen-treated mice, with the biggest difference found in the 40 mg/kg caffeine group. Therefore, we also determined brain concentrations of caffeine and its metabolites after intraperitoneal injection of caffeine at 40 mg/kg (Fig. 5*B*). There was no difference in brain caffeine concentrations between OVX+E and OVX+P mice. There was a small effect of estrogen on brain paraxanthine concentrations, which were slightly but significantly higher in OVX+P than in OVX+E mice at 120, 180, and 360 min. There was also a minor effect on brain theophylline concentrations, which were slightly higher in OVX+P than OVX+E mice 60 min after caffeine in-

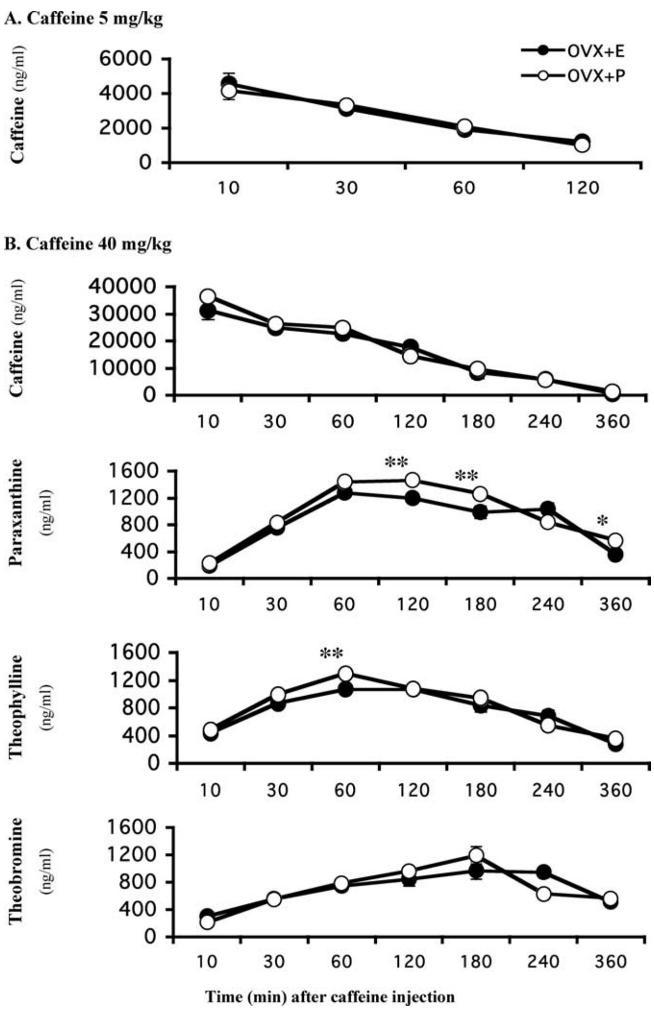

Figure 5. Caffeine metabolism is not changed by estrogen replacement in OVX retired breeder female mice. Ten days after ovariectomy, mice were implanted with placebo or estrogen pellets. Caffeine (5 or 40 mg/kg, i.p.) was administered 10 d after pellet implantation. Brain concentrations of caffeine and its metabolites were measured at 10, 30, 60, 120, 180, 240, or 360 min after injection ($n = 5$ for each time point). ***A***, Brain caffeine concentration after intraperitoneal 5 mg/kg caffeine. The levels of metabolites are below the detection limit. ***B***, Brain concentrations of caffeine, paraxanthine, theophylline, and theobromine, respectively, after intraperitoneal 40 mg/kg caffeine. Data were analyzed by two-way ANOVA, followed by Fisher's LSD test. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the respective estrogen-treated group.

jection. There was no difference in brain theobromine concentrations after caffeine injection. Thus, exposure to estrogen, under conditions that attenuated the neuroprotective effect of caffeine, had no effect on brain concentrations of caffeine. Moreover, the overall concentrations of the three caffeine dimethyl metabolites even when combined are much (an order of magnitude) lower than that of caffeine such that the slight differences in metabolite concentrations found at some time points are not sufficient to explain the difference in caffeine neuroprotection between OVX+E and OVX+P mice.

Discussion

The present data reveal a novel interaction between estrogen and caffeine in a mouse model of PD, an interaction that recapitulates the relationship between estrogen and caffeine exposures in the human epidemiology of PD. In male but not female mice, low doses of caffeine attenuated MPTP-induced depletion of striatal dopamine and DOPAC content, functional markers of the nigrostriatal neurons that degenerate in PD. Remarkably, neuroprotection by low doses of caffeine was abolished in the presence of estrogen (either endogenous or exogenous) in young and retired breeder OVX female mice. Moreover, estrogen placement in male mice effectively replicated the female phenotype of attenuated neuroprotection by caffeine.

Estrogen treatment by itself protects against MPTP toxicity

Our data, in agreement with previous reports (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998), demonstrated that MPTP induced greater striatal dopamine loss in male than female mice. These experimental findings are consistent with epidemiological studies, which have generally demonstrated a higher prevalence of PD in men than women (Dluzen et al., 1998; Baldereschi et al., 2000; Ascherio et al., 2001, 2004). The gender differences observed in PD patients and animal models support the possibility that estrogen may play a protective role in dopaminergic neurodegeneration. Indeed, our current data confirm the neuroprotective effect of estrogen replacement therapy in this mouse model of PD (Dluzen et al., 1996; Callier et al., 2001; Ramirez et al., 2003).

Attenuation of MPTP toxicity by caffeine is diminished in the presence of estrogen

The current data confirm our previous findings (Chen et al., 2001; Xu et al., 2002a,b) that caffeine dose-dependently attenuates MPTP-induced striatal dopamine loss in male mice. Moreover, our data demonstrate that this phenomenon persists as animals age. These findings support a biological basis for a causal epidemiological correlation between caffeine consumption and reduced risk of developing PD.

Strikingly, the protection by lower doses of caffeine against MPTP-induced dopamine depletion was lost in female mice. The gender difference in the dose–response relationship of caffeine with neuroprotection can be described pharmacologically as a reduction in the potency of caffeine in female compared with male mice. In both young and retired breeder mice (Fig. 1), an ED_{50} value of $\leq$5 mg/kg is apparent in males compared with $\geq$20 mg/kg in females. As we discussed above, female mice are less vulnerable than males to MPTP toxicity, raising the possibility that attenuated protection by caffeine in females may also reflect a "ceiling effect" rather than reduced potency. However, the loss of protection in both moderate and severe lesions together with repeatedly significant protection achieved by the highest dose of caffeine in female mice of different ages argue against an impenetrable ceiling and support a lower potency in females. The current data correlate well with the epidemiological studies that showed no inverse association between caffeine or coffee intake and risk of PD in women (Benedetti et al., 2000; Ascherio et al., 2001). The neuroprotection observed in female mice was restricted to the highest caffeine dose of 40 mg/kg and may not be relevant to PD epidemiology. This dose in rodents may be comparable to a human exposure to the caffeine in some 10 cups of coffee (Fredholm et al., 1999), which exceeds the daily consumption of almost any human subject in PD epidemiology studies.

The most notable finding of the current study is that the presence of the major female steroid hormone estrogen prevents neuroprotection by caffeine (or reduces its potency) in female and male mice. The estrogen effects observed in young mice were more robust in mice past their reproductive prime, highlighting the relevance of these findings to epidemiological studies linking PD risk to caffeine and estrogen exposures of middle-aged adults. These data systematically demonstrate that estrogen treatment attenuates the protection of caffeine against MPTP toxicity in this mouse model of PD, providing a possible biological basis for the epidemiological findings that an interaction between estrogen and caffeine exposure modulates the risk of PD (Ascherio et al., 2003, 2004).

Mechanism of interaction between caffeine and estrogen

In pursuing the mechanism, a pharmacokinetic or metabolic interaction is an important consideration. Human data (Patwardhan et al., 1980; Abernethy and Todd, 1985; Pollock et al., 1999) have demonstrated that caffeine metabolism is inhibited in women taking estrogen-containing oral contraceptives or estrogen replacement therapy after menopause, suggesting that the actions of caffeine would be enhanced rather than attenuated by estrogen. However, our data show no difference in brain caffeine concentrations over the time course (extending beyond three half-lives) of measurement in OVX retired breeder female mice treated with either estrogen or placebo.

Interestingly, two metabolites of caffeine (paraxanthine and theophylline) have been shown to provide protection against MPTP-induced dopaminergic toxicity with potencies comparable to caffeine (Xu et al., 2002b). So paradoxically, delaying or inhibiting the metabolism of caffeine could result in a decreased level of these metabolites, which might cause reduced protection against neurotoxicity after estrogen therapy in human or animals. In the present study, however, although there were slight differences in the brain concentrations of paraxanthine and theophylline between estrogen- and placebo-treated mice, the levels of these metabolites were much lower (>25 times) than that of caffeine, therefore arguing against their involvement.

It is interesting that although both estrogen and caffeine individually protect nigrostriatal dopaminergic neurons, when combined, estrogen diminished the potency (rather than enhancing the efficacy) of the neuroprotective action of caffeine. This finding suggests that these two agents may work through a common mechanism to prevent MPTP toxicity. Estrogen may compete with caffeine for its activation of a protective pathway, effectively "occluding" the pathway and shifting the dose–response curve of caffeine for neuroprotection to the right. It is worth noting that estrogen and caffeine as well as MPTP share a cytochrome P450, CYP1A2, for their metabolism or detoxification (Gu et al., 1992; Tassaneeyakul et al., 1994; Yamazaki et al., 1998; Forsyth et al., 2000). Therefore, metabolism of caffeine and/or estrogen could alter MPTP metabolism. However, because CYP1A2 contributes to hepatic detoxification of MPTP (Forsyth et al., 2000), competition for this enzyme by caffeine or estrogen would be expected to exacerbate rather than ameliorate MPTP toxicity. Moreover, because the brain MPTP levels were unaffected by acute caffeine treatment (Chen et al., 2001), it is unlikely that estrogen modulates the protection of caffeine through its action on MPTP metabolism. Alternatively, estrogen may noncompetitively inhibit the neuroprotective effect of caffeine while it provides its own independent protection effect. Our findings that higher doses of caffeine generally produce additional protection, however, argue against a simple noncompetitive inhibition.

Caffeine at the doses used in the present study, which produced peak brain concentrations of ~4–40 μg/ml, most likely functions as an antagonist of adenosine A_1 and A_{2A} receptors (Fredholm et al., 1999). Our previous study (Chen et al., 2001) indicates that the attenuated neurotoxicity of caffeine likely involves its antagonism at the A_{2A} receptor ($A_{2A}R$). Therefore, estrogen might directly modulate the neuroprotection by caffeine through an action on the $A_{2A}R$, although there is no information currently on such an interaction. It is reported (Rose'Meyer et al., 2003) that $A_{2A}R$ expression is significantly decreased after OVX. However, we found no difference in striatal $A_{2A}R$ binding densities between OVX mice treated with estrogen and placebo (data not shown). Among the potential mechanisms for neuroprotection by caffeine (and more specific A_{2A} antagonists (for review, see Xu et al., 2005), the attenuation of glutamate release and of resultant excitotoxicity may be a protective mechanism shared with estrogen (O'Neill et al., 2004; Ritz et al., 2004; Saleh et al., 2004). Interestingly, it has been reported that estrogen can alter dopamine D_2 receptor (D_2R)/G-protein coupling (Levesque and Di Paolo, 1993; Thompson and Certain, 2005) and D_2R affinity (Di Paolo et al., 1988). Because it is well known that A_{2A}Rs colocalize with D_2Rs in the basal ganglia and interact with each other closely (for review, see Xu et al., 2005), it is possible that estrogen could modulate $A_{2A}R$ through its action on D_2R. Whether these or other shared mechanisms of neuroprotection by caffeine and estrogen can account for their interaction in the MPTP model and in the epidemiology of PD remains to be clarified.

Relevance of an estrogen–caffeine interaction for PD

The attenuation of the neuroprotective effect of caffeine by estrogen in the MPTP model of PD establishes a potential neural basis for the epidemiological association between estrogen replacement therapy, caffeine consumption, and risk of developing PD in women. This convergence of laboratory and human epidemiological findings strengthens the contention that widely used exogenous estrogen, which may provide neuroprotection by itself, interacts with the nearly ubiquitously consumed A_{2A} antagonist caffeine to modify the risk of developing PD. The present study also establishes an animal model of this interaction in humans, along with the opportunity to pursue its molecular mechanism(s). In addition, the cumulative evidence for this interaction is sufficiently compelling to influence the design and interpretation of neuroprotection trials of estrogen or caffeine currently underway or under consideration (Ravina et al., 2003). Ultimately, a better understanding of the interplay between environmental factors like caffeine and estrogen may suggest effective preventative as well as therapeutic strategies for this neurodegenerative disorder.

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Appendix B

Review TRENDS in Neurosciences Vol.29 No.11

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Targeting adenosine A_{2A} receptors in Parkinson's disease

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The adenosine A_{2A} receptor has emerged as an attractive non-dopaminergic target in the pursuit of improved therapy for Parkinson's disease (PD), based in part on its unique CNS distribution. It is highly enriched in striatopallidal neurons and can form functional heteromeric complexes with other G-protein-coupled receptors, including dopamine D_2, metabotropic glutamate $mGlu_5$ and adenosine A_1 receptors. Blockade of the adenosine A_{2A} receptor in striatopallidal neurons reduces postsynaptic effects of dopamine depletion, and in turn lessens the motor deficits of PD. A_{2A} antagonists might partially improve not only the symptoms of PD but also its course, by slowing the underlying neurodegeneration and reducing the maladaptive neuroplasticity that complicates standard 'dopamine replacement' treatments. Thus, we review here a prime example of translational neuroscience, through which antagonism of A_{2A} receptors has now entered the arena of clinical trials with realistic prospects for advancing PD therapeutics.

Introduction

Current pharmacotherapy for Parkinson's disease (PD) can be accurately described as both highly effective and largely inadequate. On the one hand, the dramatic efficacy of standard anti-parkinsonian drugs can be the stuff of Hollywood movies, as when the body of an immobilized patient played by Robert De Niro is 'awakened' by L-dopa in the 1990 film *Awakenings*. The characteristic bradykinesia (slowness), rigidity and tremor of PD are primarily due to an underlying degeneration of dopaminergic nigrostriatal neurons and the resultant depletion of striatal dopamine. Repleting endogenous dopamine using its precursor L-dopa and mimicking it using dopamine agonists constitute 'dopamine replacement' strategies – the mainstay of current treatment. By boosting dopamine-mediated transmission, these strategies can dramatically (albeit partially) alleviate the motor deficits in PD.

On the other hand, none of the currently approved anti-parkinsonian agents has been found to alter the underlying degeneration of dopaminergic neurons. Thus, after several years of gratifying improvement using dopamine-replacement therapy, PD patients typically experience great frustration as neuronal loss and symptoms inexorably progress. As the disease advances, disability is often compounded further by the development of L-dopa-triggered involuntary jerking and writhing movements known as dyskinesias. Eventually, most will also suffer from non-motor complications of both the dopaminergic treatments and the disease itself; such complications include sleep disturbance, depression, dementia and psychosis.

The inadequacies and adverse effects of drugs that target the dopaminergic system have prompted a search for alternative or adjunctive approaches that can modulate basal ganglia motor circuitry with a reduced risk of side effects. Antagonists of adenosine A_{2A} receptors have recently emerged as a leading candidate class of non-dopaminergic anti-parkinsonian agents, based in part on the unique CNS distribution of the A_{2A} receptor (Figure 1a). As we review here, promising preclinical features have not only led to clinical trials of A_{2A} receptor antagonists as a novel symptomatic therapy for PD, but also raised the possibility of neuroprotective and anti-dyskinetic benefits.

A_{2A} antagonists as symptomatic anti-parkinsonian therapy

Neurochemical evidence that A_{2A} receptors functionally oppose the actions of dopamine D_2 receptors on GABAergic striatopallidal neurons [1–3] (Figure 1b,c; Box 1) raised the possibility that A_{2A} antagonists might boost the anti-parkinsonian action of dopamine-replacement strategies. Indeed, behavioral studies of hemi-parkinsonian rats, in which the dopaminergic nigrostriatal pathway had been lesioned on one side by 6-hydroxydopamine (6-OHDA), revealed that blockade of A_{2A} receptors markedly increased the number of contralateral rotations induced by a threshold dose of L-dopa or by stimulation of dopamine receptors; Fos-like immunoreactivity in the dorsal striatum and globus pallidus was also increased [4–7]. These results provided the first indication that blockade of A_{2A} receptors, by potentiating dopamine transmission, might contribute to restoring the motor impairment observed in models of PD. Importantly, in contrast to the non-specific adenosine antagonist caffeine, which can lose its motor-stimulant effect with repeated exposure, A_{2A} antagonists did not produce tolerance effects after sub-chronic treatment in PD models [8,9].

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The ability of A_{2A} antagonists to reverse parkinsonian deficits consistently in non-human primates [8,10] in addition to rodents led to clinical trials in PD patients that had encouraging, if modest, initial results [11,12] (reviewed in depth in Ref. [13]). These studies demonstrated symptomatic improvement in patients with relatively advanced PD, who had already developed dyskinetic motor complications. Pairing KW-6002, a well-characterized xanthine-based A_{2A} antagonist, with a reduced dose of L-dopa produced symptomatic relief comparable to that produced by an optimal dose of L-dopa alone but with less dyskinesia [11]. However, when paired with a full dose of L-dopa, KW-6002 improved symptoms further only under some circumstances and perhaps slightly exacerbated dyskinesia [11–13].

Like bradykinesia, two other cardinal motor features of PD might also improve with A_{2A} blockade. Muscle rigidity, manifest clinically as increased resistance to passive movement, is an early and progressive sign of PD. In rodents, muscle rigidity induced by the dopamine-depleting agent reserpine can be reduced by an A_{2A} antagonist or eliminated by a synergistic combination of L-dopa plus A_{2A} antagonist [14]. Parkinsonian rest tremor, which is relatively resistant to dopamine-replacement therapy, might also be targeted by A_{2A} antagonists. In rodent models of parkinsonian tremor, A_{2A} antagonists counteracted tremulous movements [15,16], in agreement with data from a recent clinical trial indicating that the combination of the A_{2A} antagonist KW-6002 and a subthreshold dose of L-dopa counteracted resting tremor more effectively than they did other cardinal symptoms of PD [11]. Moreover, the intrastriatal infusion of the water-soluble non-xanthine A_{2A} antagonist SCH BT2 demonstrated that, although the effects of A_{2A} antagonists on tremor are exerted throughout the whole striatum, the ventrolateral portion is the area most involved in this effect [16].

The mechanism by which A_{2A} antagonists improve parkinsonian motor dysfunction probably involves their direct inhibitory influence on striatopallidal neurons, which coexpress A_{2A} and D_2 receptors [17,18]. The blockade of A_{2A} receptors on these neurons offsets the hypolocomotor effect of lost dopamine stimulation at striatal D_2 receptors (Figure 2), accounting for much of the symptomatic

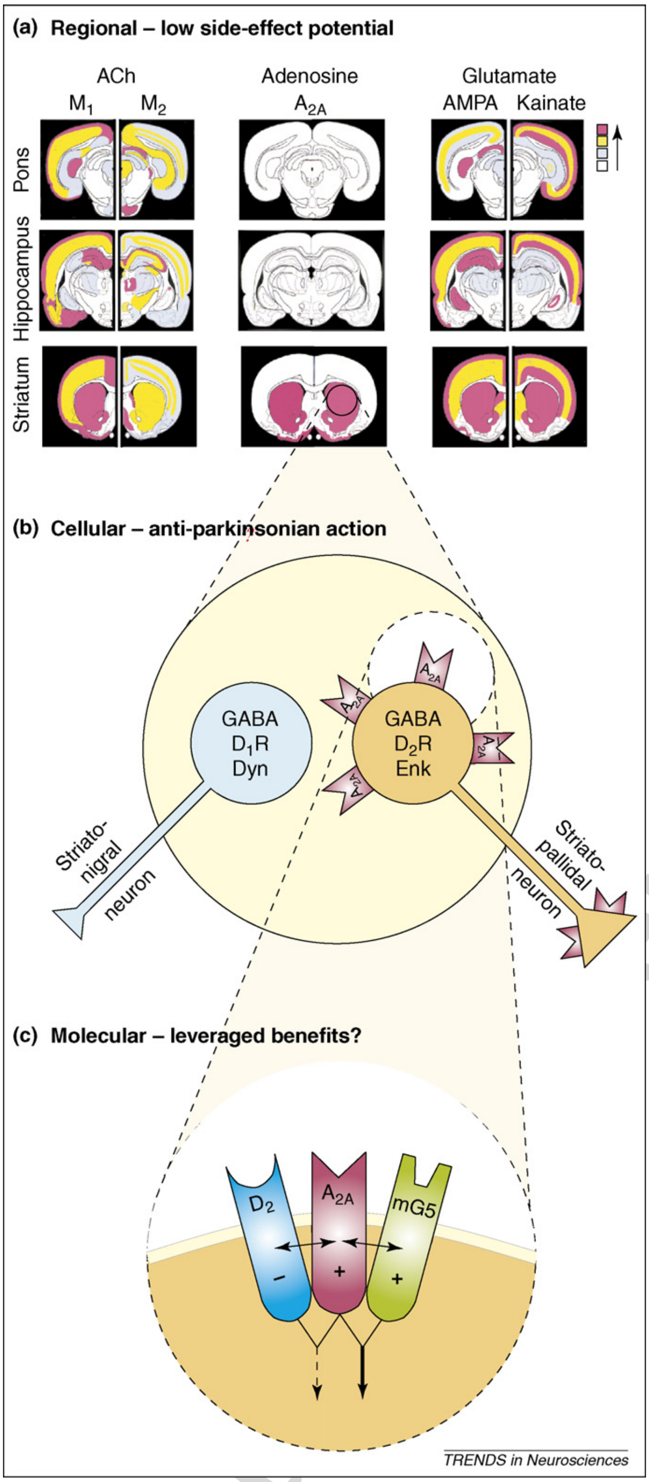

Figure 1. Unique CNS localization of adenosine A_{2A} receptors at multiple levels contributes to the therapeutic potential of A_{2A} antagonists for PD. **(a)** Regional localization of brain A_{2A} adenosine receptors versus other non-dopamine receptors that are targeted in PD: the basis for the relatively low risk of CNS side effects from A_{2A} antagonists. Brain expression patterns are shown for subtypes of receptors for three neurotransmitters that modulate dopamine-mediated neurotransmission. A_{2A} receptors are highly enriched in the striatum, with very low (but possibly functionally significant) levels in the cortex and other brain regions. By contrast, other receptors targeted by current non-dopamine anti-parkinsonian drugs are widely distributed in the brain. Agents that block muscarinic ACh receptors and amantadine, which blocks the function of ionotropic glutamate receptors, improve PD symptoms but are often intolerable owing to cognitive and other CNS side effects, as would be expected from the broad expression of these receptors in the cortex and other extra-striatal brain regions. Increasing density of specific radioligand binding to each receptor subtype is represented by the color scale on the far right, and is shown in coronal sections from the caudal, mid and rostral rat brain (at the levels of the pons, hippocampus and striatum, respectively). Adapted, with permission, from Refs [13,73]. **(b)** Cellular localization of A_{2A} receptors within the striatum: the basis for anti-parkinsonian action of A_{2A} antagonists. In the striatum, A_{2A} receptors are largely restricted to GABAergic neurons that express dopamine D_2 receptors (D_2R) and enkephalin (Enk) [17,18] and project to the globus pallidus. By contrast, the striatal GABAergic neurons that project to the substantia nigra are enriched for D_1 receptors (D_1R) and dynorphin (Dyn) but do not appreciably express A_{2A} receptors. Inhibition of the striatopallidal neurons by A_{2A} antagonism likely reduces motor deficits caused by dopamine deficiency in PD (Figure 2). **(c)** Molecular environment of A_{2A} receptors: a possible opportunity for leveraging anti-parkinsonian effects. A_{2A} receptors activate adenylate cyclase and generally stimulate neuronal activity. They can form heteromers with inhibitory (G_i-coupled) D_2 receptors [55,56,58] and stimulatory metabotropic glutamate $mGlu_5$ receptors (mG5) [65] to attenuate (thin broken downward arrow) or amplify (thick solid downward arrow) their respective signaling pathways. Functional heteromeric interactions can be direct (bidirectional arrows) or indirect (through downstream signaling, as indicated), and can open new avenues for enhancing anti-parkinsonian actions of A_{2A} antagonists (Box 1). The interactions might occur postsynaptically, as schematized here on striatopallidal neurons, or presynaptically to modulate neurotransmitter release [66].

Box 1. Receptor heteromerization: a key advance in A_{2A} neurobiology

A fuller understanding of the biology of the A_{2A} receptor, particularly its molecular interactions with other neurotransmitter receptors, will refine our ability to target it for the treatment of PD and other CNS disorders. The discovery of functional heteromeric receptor complexes (receptor mosaics) comprising the A_{2A} receptor and other G-protein-coupled receptors (Figure 1c of the main text) constitutes a major advance in adenosine neurobiology and creates new opportunities to leverage the anti-parkinsonian actions (both symptomatic and disease-modifying) of A_{2A} antagonists [52,53].

A_{2A}–D_2 heteromers

Coimmunoprecipitation studies have demonstrated the existence of constitutive A_{2A}–D_2 complexes at the sites of colocalization [52,54,55]. Direct physical evidence for A_{2A}–D_2 heteromers in addition to A_{2A} homodimers within the plasma membrane came from fluorescent and bioluminescent resonance energy transfer (FRET and BRET) analyses, indicating that <10 nm separates the receptors [56–58]. The stoichiometry of the A_{2A}–D_2 heteromers remains unknown and they could be dimers or higher-order A_{2A}–D_2 hetero-oligomers, which might help to explain antagonistic membrane-level (and ultimately behavioral) interactions between the A_{2A} and D_2 receptors. For example, an A_{2A}–D_2 trimer containing a D_2 receptor dimer and an A_{2A} monomer could account for the observed increase in the K_d value of D_2 agonist binding sites if A_{2A} monomer activation were to enhance negative cooperativity between the dimeric D_2 components [53,54]. A_{2A} receptor activation can produce this direct molecular inhibition of D_2 receptors and might also reduce D_2 coupling to G_i [52], suggesting that molecular interactions between A_{2A} and D_2 receptors are important for the anti-parkinsonian actions of A_{2A} antagonists. However, the fact that A_{2A} antagonists improve parkinsonian motor deficits in the absence of the D_2 receptor (albeit to a lesser extent than in its presence) [59,60] indicates that a mechanism other than A_{2A}–D_2 heteromerization also contributes to their anti-parkinsonian properties.

A_{2A}–D_3 heteromers

Similarly, evidence for functional A_{2A}–D_3 heteromers has recently been obtained in cells cotransfected with A_{2A} and D_3, using FRET and by activating A_{2A} receptors, which reduced both the affinity of D_3 agonist-binding sites and D_3 signaling [61]. Because D_3 dimers and tetramers exist in the brain [62], one can envisage the existence of higher-order A_{2A}–D_3 hetero-oligomers, in which cooperativity between multimeric D_3 components might be modulated by an A_{2A} receptor within the receptor mosaic.

A_{2A}–$mGlu_5$ heteromers

A_{2A} receptors have also been found to partner with non-dopamine receptors – most notably the metabotropic glutamate receptor $mGlu_5$, which itself is candidate target for new symptomatic and neuroprotective anti-parkinsonian therapies. Evidence that A_{2A} and group I mGlu receptor agonists, including $mGlu_5$ agonists, could synergistically reduce the affinity of the D_2 agonist-binding sites in striatal membranes [63,64] was followed by physical (coimmunoprecipitation) evidence that A_{2A} and $mGlu_5$ form heteromeric receptor complexes [65]; such complexes might explain the synergy between A_{2A} and $mGlu_5$ antagonists. This was supported by observations of a high degree of A_{2A} and $mGlu_5$ colocalization in striatal neurons in primary cultures [52] (Figure Ia of this box), and more recently in glutamatergic nerve terminals in the striatum [66]. Coactivation of the A_{2A} and $mGlu_5$ receptors caused a synergistic interaction at the level of *c-fos* expression and phosphorylation of extracellular signal regulated kinase (ERK) and dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), indicating a role for A_{2A}–$mGlu_5$ complexes in striatal plasticity [65,67]. Combined A_{2A} and $mGlu_5$ receptor activation can also produce synergistic cellular effects on striatal output neurons *in vivo* (Figure 1c of the main text), as demonstrated by a greater than additive increase in GABA release from ventral striatopallidal neurons after local perfusion of both A_{2A} and $mGlu_5$ agonists (Figure Ic of this box) [68].

A_{2A}–D_2–$mGlu_5$ heteromers as therapeutic targets for PD

The discovery of heteromeric A_{2A}–D_2 complexes has added to the substantial evidence for antagonistic molecular, cellular, electrophysiological and behavioral interactions between A_{2A} and D_2 receptors, and strengthens the rationale for anti-parkinsonian strategies that simultaneously block adenosine A_{2A} receptors and stimulate dopamine (D_1 and D_2) receptors [52,54]. In retrospect, the initial clinical efficacy of A_{2A} antagonism when tested as an adjunct to standard dopamine therapy (but not when tested as a monotherapy) in relatively advanced PD [11] might reflect the benefits of dual targeting. Notably, one might predict the greater likelihood of success using A_{2A}-antagonist monotherapy in earlier disease, when higher levels of residual endogenous dopamine provide greater tonic D_2 receptor activation.

Similarly, the discovery of functional A_{2A}–$mGlu_5$ interactions and heteromeric complexes catalyzed research into the synergistic anti-parkinsonian potential of combining A_{2A} and $mGlu_5$ antagonists [69,70] (Figure Ic of this box). The additional finding that $mGlu_5$-antagonist-induced motor activation requires A_{2A} and D_2 receptors [70] highlights the interdependence of these three receptors in modulating motor function. It also supports the concept of A_{2A}–D_2–$mGlu_5$ receptor mosaics [52,71]. These might integrate multiple signals converging on striatopallidal GABAergic neurons, and suggests future opportunities for leveraging the anti-parkinsonian actions of A_{2A} antagonists.

Lastly, there is now physical and functional evidence that heteromeric A_1–A_{2A} receptors can form both in receptor-overexpressing cell lines and in the striatum [72]. This study suggests that, just as in A_{2A}–D_2 receptor interactions, negative cooperativity could explain why A_{2A} receptor stimulation might reduce A_1 receptor binding of adenosine, and thus reduce A_1-mediated inhibition of presynaptic glutamate release. Conversely, through such an interaction an A_{2A} antagonist might increase binding of adenosine to its A_1 receptor, and thus amplify the inhibition of glutamate release, potentially alleviating excitotoxic injury to dopaminergic neurons.

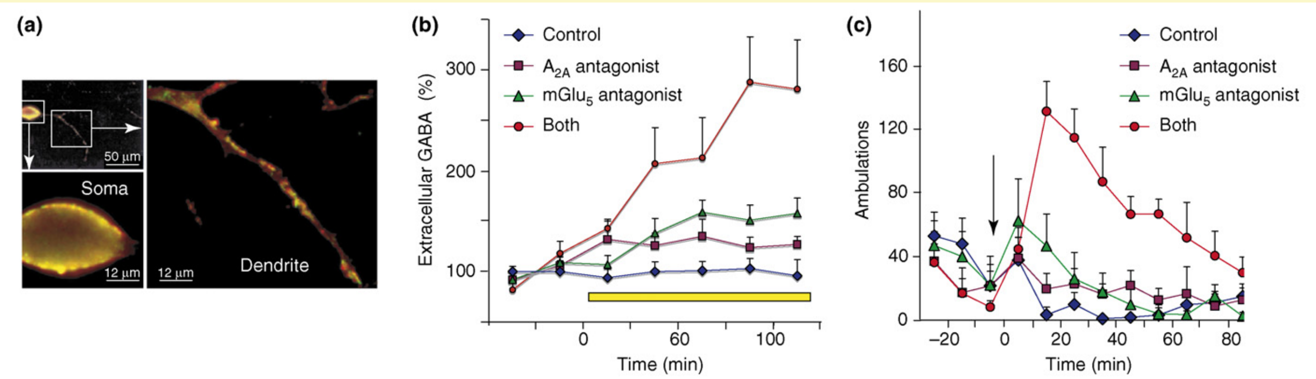

Figure I. A_{2A}–$mGlu_5$ receptor interactions in striatal neurons: anatomical, neurochemical and antiparkinsonian features. (a) A_{2A} (green) and D_2 (red) receptor immunoreactivities are colocalized (yellow) and primarily associated with the plasma membrane in cell bodies and dendrites of striatal neurons in primary cultures.

Adapted, with permission, from Ref. [52]. **(b)** Combined activation of A_{2A} and $mGlu_5$ receptors can synergistically activate striatopallidal neurons *in vivo*, as shown by the synergistic elevation of extracellular GABA levels (measured as a percentage of the average initial two baseline determinations) in the ventral pallidum of awake, freely moving rats after perfusion of both an A_{2A} and an $mGlu_5$ agonist (CGS 21680 and CHPG, respectively) into the ventral striatum (indicated by the horizontal yellow bar). Adapted from Ref. [68]. **(c)** Combined antagonism of A_{2A} and $mGlu_5$ receptors can synergistically enhance anti-parkinsonian locomotor stimulation in hypodopaminergic, hypolocomotor mice (treated one day earlier with 1 mg kg^{-1} reserpine). Adapted from Ref. [70]. Mice were treated systemically (arrow) with control vehicles, an A_{2A} antagonist (intraperitoneal KW-6002 at 0.3 mg kg^{-1}), an mGlu5 antagonist (intraperitoneal MPEP at 5 mg kg^{-1}) or both.

anti-parkinsonian benefit of A_{2A} antagonism. However, this relatively selective targeting of the D_2-modulated 'indirect' pathway (Figure 2 legend) might also explain why the symptomatic effect of an A_{2A} antagonist on its own appears modest compared with that of L-dopa (which can restore stimulation at all dopamine receptors). Evidence for this A_{2A} receptor mechanism includes the correlation between the anti-parkinsonian effects of A_{2A} antagonists and their ability to modulate GABA release and dopamine-dependent *c-fos* activation specifically in the indirect striatopallidal pathway [19,20].

A_{2A} antagonists as potential neuroprotectants in PD

Over past six years, converging epidemiological and experimental evidence has raised the exciting possibility that A_{2A} receptor antagonism might protect dopaminergic neurons from degeneration in PD [13,21]. In 2000, Ross and colleagues reported an inverse relationship between the consumption of caffeine (1,3,7-trimethylxanthine), a non-specific adenosine receptor antagonist, and the risk of developing PD in 8004 Japanese–American men followed for 30 years as part of a prospective study [22]. The risk of PD adjusted for age and smoking was five times higher among men who reported no coffee consumption than among men who reported a daily consumption of 28 ounces of coffee or more. The finding was substantiated by a similar inverse relationship between the consumption of caffeinated (but not decaffeinated) coffee and the risk of developing PD in a larger and more ethnically diverse cohort of prospectively followed men [23]. Curiously, in women caffeine use is also linked to a reduced risk of PD but only among those who have not taken hormone-replacement therapy [24].

The neuroprotective potential of caffeine was further enhanced by genetic and pharmacological evidence that the A_{2A} receptor can contribute to the degeneration of nigrostriatal dopaminergic neurons [13,21,25]. Caffeine at doses in mice corresponding to those of typical human exposure, which lead to blockade of adenosine A_1 in addition

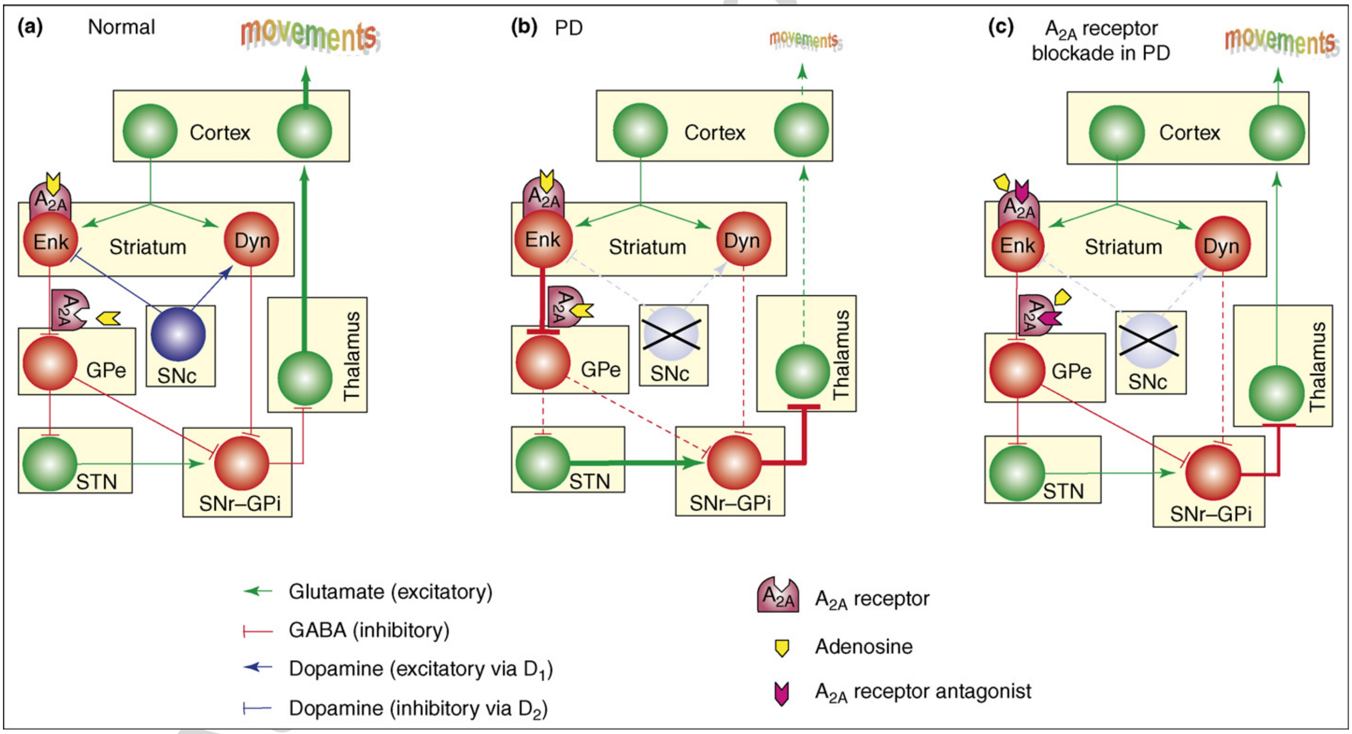

Figure 2. Proposed mechanism of symptomatic anti-parkinsonian activity of A_{2A} receptor antagonists. The striatum is linked to the substantia nigra pars reticulata and globus pallidus pars interna complex (SNr–GPi) via direct (striatonigral) and indirect (striatal–pallidal–subthalamic–nigral) pathways. According to evolving models of basal ganglia organization and function [74], in the 'normal' state **(a)**, dopamine (blue) from neurons of the substantia nigra pars compacta (SNc) acts on stimulatory D_1 receptors of the direct striatonigral pathway and on inhibitory D_2 receptors of the indirect pathway to facilitate intricate and rapid movements. Adenosine, via A_{2A} receptors on striatopallidal neurons in the striatum and globus pallidus pars externa (GPe), excites neurons in the indirect pathway, thus opposing the activation of D_2 receptors. **(b)** Degeneration of the SNc in Parkinson's disease (PD) removes dopamine input to the striatum. This disinhibits striatal spiny projection neurons of the indirect pathway, boosting their inhibitory GABA-mediated (red) influence on the GPe, which in turn leads to disinhibition of the excitatory glutamate-mediated (green) transmission of the subthalamic nucleus (STN). Depletion of dopamine also leads to decreased activation of striatal spiny neurons in the direct pathway. The resulting imbalance between the activity in the direct and the indirect pathways leads to markedly increased inhibitory output from the SNr–GPi complex. The subsequent excessive inhibition of thalamocortical neurons produces the characteristic reduction of movements of PD. **(c)** A_{2A} receptor blockade in PD should relieve overactivity of striatopallidal and subsequently STN neurons, thereby restoring some balance between the direct and the indirect pathways. Note that an A_{2A} antagonist together with a low dose of L-dopa (which elevates extracellular levels of dopamine that stimulates both D_1 and D_2 receptors) would facilitate the inhibitory cellular action of D_2 receptors on striatopallidal neurons. In turn, through the basal ganglia circuitry or collaterals from striatal medium-spiny neurons [75], this might indirectly facilitate activation of the striatonigral pathway stimulated by D_1 receptors [4] (not shown). Adapted, with permission, from Ref. [13] and based on references therein.

to A_{2A} receptors in the brain, dose-dependently attenuates the loss of striatal dopaminergic terminals and nigral dopaminergic neurons that is triggered by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [13,25]. This protective effect of caffeine was also observed with several relatively selective antagonists of A_{2A} (but not A_1) receptors, including KW-6002 [13,26]. Similarly, genetic deletion of the A_{2A} receptor can attenuate the loss of striatal dopamine and dopaminergic terminals in the mouse MPTP model [25]. Interestingly, neuroprotection by caffeine, in contrast to its motor stimulant effect, does not exhibit tolerance after repeated prior injection [27]. Another striking feature of protection by caffeine against MPTP toxicity is its attenuation or absence in the presence of estrogen [28], closely paralleling the aforementioned relationship between caffeine and female hormone replacement in PD epidemiology. These complementary pharmacological and genetic studies provide compelling evidence that caffeine and more selective A_{2A} receptor inactivation generally reduce the neurotoxic effects on dopaminergic neurons in animal models of PD neurodegeneration, although it should be noted that these laboratory models are as yet unproven as predictors of neuroprotection in humans. Thus the laboratory data suggest (but do not prove) that neuroprotection by caffeine is the basis for the reduced risk of developing PD among coffee drinkers and other caffeine consumers. It would be worthwhile to evaluate further whether the neuroprotective effect of A_{2A} antagonism extends beyond acute neurotoxin models of PD and ultimately to PD patients.

The inferred pathophysiological role of endogenous adenosine acting through A_{2A} receptors to hasten neurodegeneration is not restricted to dopaminergic neurons. A_{2A}-antagonist-mediated neuroprotection extends beyond PD models of nigrostriatal neuron degeneration. In fact, neuroprotection by A_{2A} receptor antagonism was first reported in a global ischemia model [29] and further substantiated in other models of ischemic and excitotoxic brain injury in cortical regions [30,31]; this might also be of therapeutic relevance to A_{2A} antagonist use in PD, because in its later stages cortical and basal ganglia neurodegeneration both contribute to the disease. In addition, A_{2A} receptor antagonists can limit the damage to striatal output neurons that is induced by mitochondrial toxins [32–34], and can reduce formation of β-amyloid-induced aggregations in cultured cells [35], in models of both Huntington's disease and Alzheimer disease. However, A_{2A} receptor inactivation has been found both to exacerbate and to alleviate the death of striatal neurons under different conditions, possibly owing to differential postsynaptic and presynaptic actions of the A_{2A} receptor in striatum [32]. This suggests that any neuroprotective benefits of A_{2A} antagonists in humans might apply to only a subset of degenerative CNS diseases.

The mechanism by which A_{2A} receptor antagonists confer protection against death of dopaminergic neurons is not clear at present. However, the fact that their neuroprotective effects extend to other types of neurons in the basal ganglia and cerebral cortex supports a broad-based CNS mechanism that acts through common cellular elements – neuronal, glial and/or immunological. For example, A_{2A} receptor activation on widely distributed glutamatergic nerve terminals or astrocytes might enhance glutamate release and thereby contribute to an 'excitotoxic' component of neuronal cell death [36–38]. Alternatively, A_{2A} receptors on microglia and other immune cells might facilitate neuroinflammation that has an important role in brain injury [39,40], consistent with a recent report that neuroprotection against MPTP neurotoxicity by KW-6002 is associated with inhibition of microglial activation in the substantia nigra [41]. A better understanding of how the multiple actions of A_{2A} receptors influence survival of dopaminergic neurons might further establish A_{2A} antagonism as a potential neuroprotective strategy for the treatment of PD.

A_{2A} receptors in dyskinesias and non-motor targets

With the progressive loss of dopaminergic neurons in PD come other disabling motor and behavioral problems. Chronic intermittent therapy using L-dopa (or a dopamine agonist) can conspire with the hypodopaminergic state of PD to produce progressively briefer motor benefits and progressively more disruptive involuntary movements (dyskinesias) in response to each dose. The prevention and suppression of L-dopa-induced dyskinesias (LID) have become major targets of new non-dopamine approaches.

Although early clinical data suggest that A_{2A} antagonists do not suppress LID after it is established [11,12], preclinical findings raise the possibility that A_{2A} blockade can reduce the risk of developing LID in the first place. Hemi-parkinsonian rats that are treated daily with L-dopa develop a markedly sensitized rotational response over weeks. When an A_{2A} antagonist was co-administered with a low dose of L-dopa, the acute effect of the combination matched that of a higher dose of L-dopa alone. By contrast, chronic use of the combined treatment caused no sensitization [9], supporting a rationale for combining A_{2A} antagonists with a low dose of L-dopa earlier in the disease.

In addition to reducing the risk of LID by enabling use of a reduced dose of L-dopa, A_{2A} antagonists might protect against LID more directly, by disrupting the pathophysiology of LID. A_{2A} receptors are crucial in the development of a persistent sensitized motor response to L-dopa in parkinsonian mice, because their A_{2A} knockout littermates did not develop this response [42]. Similarly, in parkinsonian rats repeatedly treated with L-dopa to model the motor complications of its chronic use in PD, pairing an A_{2A} antagonist with L-dopa prevented shortening of the duration of the rotational response [43]. However, in another model, in which LID is represented by abnormal involuntary movements rather than enhanced rotational behaviors, A_{2A} antagonism showed no attenuating effect on the development of these movements [44]. This highlights the uncertainty both over which rodent models of LID are most useful [45] and over the role of A_{2A} receptors in LID. Nevertheless, using the gold-standard primate model of dyskinesias, Bibbiani *et al.* found that chronic treatment using the A_{2A} antagonist KW-6002 completely prevented the induction of dyskinesias that is normally seen in parkinsonian (MPTP-lesioned) cynomolgus monkeys after 1–2 weeks of daily administration of a dopamine receptor agonist [43]. Most striking was their observation that continuing this treatment following cessation of adjunctive KW-6002 administration led to the induction of dyskinesias with the same 1–2 week lag time, strengthening the rationale for a trial of early A_{2A} antagonist treatment as an adjunct to L-dopa.

Although mechanisms of dyskinesia are poorly understood, the chronic administration of L-dopa has been clearly correlated with features of dyskinesia such as the long-term increase in striatal dynorphin mRNA, the modified phosphorylation state of NMDA and AMPA receptor subunits, and inhibition of activity in the substantia nigra pars reticulata [43,46–49]. By contrast, daily L-dopa administered in combination with an A_{2A} antagonist or in the absence of A_{2A} receptors did not trigger these modifications (and might attenuate the increase in enkephalin mRNA levels that is induced by 6-OHDA), providing possible mechanisms for the low dyskinetic potential observed with this drug combination [13,42,43,47,48].

Clinical development of A_{2A} antagonists as anti-parkinsonian agents should also take into account evidence for A_{2A} receptor involvement in common non-motor CNS disorders in PD patients, such a depression, psychosis, dementia and disrupted arousal and sleep states. For example, because A_{2A} antagonists might have anti-depressant properties [50], it will be important to consider whether motor and subjective improvement reflects elevation of mood, which is commonly depressed in PD. Whereas an antidepressant side benefit could enhance their therapeutic impact, A_{2A} antagonists also carry theoretical risks. For example, the possibility of a pro-psychotic side effect of A_{2A} antagonists has been raised, given their demonstrated ability to enhance transmission by D_2 receptors and the well-known pro-psychotic effects of D_2 agonists. Lastly, the well-established peripheral effects of A_{2A} receptors [51], particularly their anti-inflammatory actions, must also be addressed in the clinical development of A_{2A} antagonists, especially in advanced PD patients who are at higher risk of and from infections.

A_{2A} antagonists for PD: lackluster or blockbuster?

This question may seem overly theatrical, with 'lackluster' too pessimistic given the positive results of initial clinical trials using A_{2A} antagonists for PD, and 'blockbuster' too optimistic given that this label is conventionally reserved for conditions even more prevalent than PD. Nevertheless, the possibilities for A_{2A} antagonism in PD are currently wide open. On the one hand, more than the initially demonstrated mild symptomatic benefit of adjunctive A_{2A} antagonists will probably be needed to have a major effect on how we treat PD. On the other hand, A_{2A} antagonists might offer disease-modifying and more substantial symptomatic benefits. So although the promise of these compounds is counterbalanced by the long odds inherent in clinical drug development, A_{2A} antagonism clearly offers a uniquely hopeful and realistic opportunity for improving PD treatment.

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Appendix C

P-30

May 17 - 19, 2006
Boston USA

Targeting Adenosine A_{2A} Receptors in Parkinson's Disease and Other CNS Disorders

presented by
MassGeneral Institute for Neurodegenerative Disease

The neuroprotection by caffeine in the MPTP model of Parkinson's disease is lost in adenosine A_{2A} knockout mice.

Kui Xu[1]*, Yue-Hang Xu[1], Jiang-Fan Chen[2], Michael A. Schwarzschild[1]

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Background: Prospective epidemiological studies have raised the possibility of caffeine conferring protection against Parkinson's disease. This hypothesis is strengthened by our previous findings that caffeine attenuates MPTP-induced dopaminergic neurotoxicity in mice. Moreover, antagonists of the A_{2A} subtype of adenosine receptor ($A_{2A}R$), but not of the A_1R, provided similar protection. To further investigate the dependence upon and location of the $A_{2A}R$ in caffeine's neuroprotection, we examined the effect of caffeine on MPTP neurotoxicity in standard (global) $A_{2A}R$ knockout (A_{2A} KO) mice as well as tissue-specific (conditional) A_{2A} KO mice.
Methods: Postnatal forebrain neuron-specific and astrocyte-directed conditional A_{2A} KO mice were generated by using the Cre-*loxP* system based on the specificity of *CamKIIα* and *GFAP* gene promoters, respectively. Tissue-specific disruption of the $A_{2A}R$ was confirmed by PCR and western blot. Locomotion, scored as the number of adjacent photobeam breaks (*Ambulation*), was determined 3 hr before and 3 hr after caffeine/saline injection in wide-type and knockout mice. In neuroprotection experiment, caffeine or saline were administered 10 minutes before MPTP treatment (40 mg/kg ip single injection). One week later, striatal dopamine content was determined by HPLC.
Results: Caffeine-stimulated locomotion is significantly decreased in forebrain neuron-specific $A_{2A}R$ KO mice, similar to what we found previously in global $A_{2A}R$ KO mice. MPTP treatment (40 mg/kg single injection) produced similar dopamine depletion in knockout mice and their respective wide-type littermates. On the other hand, caffeine pretreatment (25 mg/kg ip) significantly attenuated MPTP-induced striatal dopamine loss in wild-type mice. This neuroprotection by caffeine, however, is lost in global $A_{2A}R$ KO mice. Similarly, caffeine attenuated MPTP-induced dopamine depletion in control but not forebrain neuron-specific $A_{2A}R$ KO mice. On the other hand, caffeine's attenuation of MPTP neurotoxicity is present in both control and astrocyte-directed $A_{2A}R$ KO mice.
Conclusions: Taken together, these data suggest that caffeine's neuroprotection against MPTP neurotoxicity is dependent on the $A_{2A}R$, particularly those located in forebrain neurons.

Support contributed by: NIEHS, Beeson Program/AFAR & USAMRAA.

To the best of my knowledge and judgment I, the presenting author, report that:
The authors have no financial conflict of interest in the presentation of this work.

Appendix D

abstract submission form

Targeting Adenosine A_{2A} Receptors in Parkinson's Disease and Other CNS Disorders

May 17 - 19, 2006
Boston USA

presented by

MassGeneral Institute for Neurodegenerative Disease

AAV-Cre/*loxP* conditional KO of adenosine A_{2A} receptors in striatal neurons

Augusta Pisanu*, Miguel Sena-Esteves & Michael A. Schwarzschild

Department of Neurology, Massachusetts General Hospital, Charlestown, MA USA;
*Presenting author (apisanu@partners.org.)

Gene knockout (KO) approaches to receptor function complement traditional pharmacological methods by complete specificity and inactivation. However, standard KO strategies globally eliminate the targeted receptor, and thus their use for investigating the role of receptors in the adult brain can be confounded by developmental or systemic phenotypes. The transgenic Cre/*loxP* conditional KO system can achieve partial control over the timing and distribution of receptor inactivation using a specific promoter to direct *cre* expression, e.g., to study the effects of the adenosine A_{2A} receptor (A_{2A}R) in postnatal forebrain. To achieve an even higher degree of precision in eliminating the A_{2A}R from discrete regions on one side of the brain, and in pursuit of a better understanding of A_{2A}R involvement in neurodegeneration, we adopted an AAV-Cre/*loxP* system. Adeno-associated virus (AAV)-Cre vectors were stereotactically infused into homozygous *floxed $A_{2A}R$* adult mice, resulting in a conditional KO of the A_{2A}R at the site and time of infection.

Working with several serotypes of AAV-Cre and AAV-GFP, injected into the striatum of *floxed $A_{2A}R$* mice, we demonstrated an infection and GFP expression largely restricted to the targeted striatum for AAV1 serotype (Fig. A) but not AAV1/8 (which produced a widespread infection extending into the overlying cortex). Focusing on AAV1 serotype we characterized the titer-dependence and time-course of neuronal Cre expression and A_{2A}R loss, visualized by IHC (as in Fig. B). Cre expression was detectable 8 days post-infusion of AAV vectors but the loss of A_{2A}R was not evident until the 16th day, reaching a maximum extent at the 32nd day post-injection.

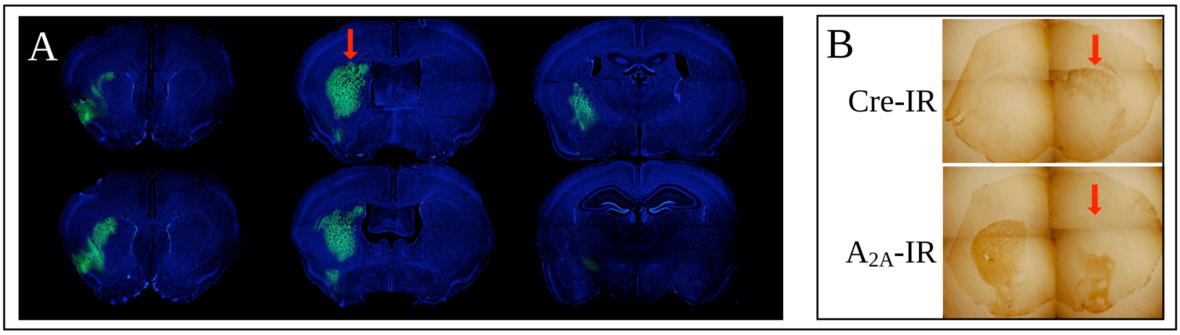

Figure: (**A**) Fluorescence of rostral→caudal coronal sections stained with a Hoechst 33258 one month after AAV1-GFP infusion (arrow). (**B**) Striatal Cre expression and the coincident elimination of striatal A_{2A}R in a *floxed $A_{2A}R$* mouse one month after unilateral intrastriatal injection of AAV1-Cre (arrow).

The AAV-Cre/*loxP* conditional KO system provides a precise tool with which to explore the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

Funded by DoD W81XWH-04-1-0881.

To the best of my knowledge and judgment I, the presenting author, report that:
[Please check one.]

☑ The authors have no financial conflict of interest‡ in the presentation of this work.

❑ The authors may have a financial conflict(s) of interest‡ as I have described on the next page.

‡ Significant *financial conflict of interest* is explained under the abstract submission policy (rule #11) and scientific communication guildelines (section 1.6) of the Society for Neuroscience.

Appendix E

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Program#/Poster#: 470.17/LL43

Title: Neuroprotection by caffeine in the MPTP model of Parkinson's disease: the role of adenosine A2A receptor

Location: Georgia World Congress Center: Halls B3-B5

Presentation Start/End Time: Monday, Oct 16, 2006, 1:00 PM - 2:00 PM

Authors: ***K. XU**[1], Y. XU[1], J. CHEN[2], M. A. SCHWARZSCHILD[1];

[1]Dept Neurol, Massachusetts Gen Hsptl, Charlestown, MA, [2]Neurology, Boston University School of Medicine, Boston, MA.

Prospective epidemiological studies have raised the possibility of caffeine conferring protection against Parkinson's disease. This hypothesis is strengthened by our previous findings that caffeine attenuates MPTP-induced dopaminergic neurotoxicity in mice. Moreover, antagonists of the A_{2A} subtype of adenosine receptor ($A_{2A}R$), but not of the A_1R, provided similar protection. To further investigate the dependence upon and location of the $A_{2A}R$ in caffeine's neuroprotection, we examined the effect of caffeine on MPTP neurotoxicity in standard (global) $A_{2A}R$ knockout (A_{2A} KO) mice as well as tissue-specific (conditional) A_{2A} KO mice. Postnatal forebrain neuron-specific and astrocyte-directed conditional A_{2A} KO mice were generated by using the Cre-loxP system based on the specificity of CamKIIα and GFAP gene promoters, respectively. Tissue-specific disruption of the $A_{2A}R$ was confirmed by PCR and western blot. Locomotion (ambulation) was determined 3 hr before and 3 hr after caffeine/saline injection. In neuroprotection experiment, caffeine or saline were administered 10 minutes before MPTP treatment (40 mg/kg ip single injection). One week later, striatal dopamine content was determined by HPLC. Caffeine's effect on MPTP-induced dopamine release will also be studied in the conditional A_{2A} KO mice using microdialysis. Caffeine-stimulated locomotion is significantly decreased in forebrain neuron-specific $A_{2A}R$ KO mice, similar to what we found previously in global $A_{2A}R$ KO mice. MPTP treatment (using the single dose injection paradigm) produced similar dopamine depletion in global KO mice and their wide-type or heterozygous littermates. By contrast, caffeine pretreatment (25 mg/kg ip) significantly attenuated MPTP-induced striatal dopamine loss in wild-type and heterozygous mice but not in global $A_{2A}R$ KO mice. Similarly, caffeine attenuated MPTP-induced dopamine depletion in control but not forebrain neuron-specific $A_{2A}R$ KO mice. On the other hand, caffeine's attenuation of MPTP neurotoxicity is present in both control and astrocyte-directed $A_{2A}R$ KO mice. Taken together, these data suggest that caffeine's neuroprotection against MPTP neurotoxicity is dependent on A_{2A} receptors, particularly those located on forebrain neurons.

Disclosures: **K. Xu** , None; **Y. Xu**, None; **J. Chen**, None; **M.A. Schwarzschild**, None.

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Beeson Program/AFAr

USAMRAA W81XWH-04-1-0881

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Appendix F

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Program#/Poster#: 199.23/OO90

Title: Conditional knock-out of striatal adenosine A2A receptors using an adeno-associated virus-Cre/loxP system

Location: Georgia World Congress Center: Halls B3-B5

Presentation Start/End Time: Sunday, Oct 15, 2006, 10:00 AM -11:00 AM

Authors: ***A. PISANU**, M. SENA-ESTEVES, M. A. SCHWARZSCHILD;
Neurology, Massachusetts General Hospital, Charlestown, MA.

Gene knockout (KO) approaches to the study of receptor function complement traditional pharmacological methods by providing complete inactivation and specificity. However, standard KO strategies globally eliminate the targeted receptor starting prenatally, and thus their use for investigating the role of receptors in the adult brain can be confounded by developmental or systemic phenotypes. The transgenic Cre/*loxP* conditional KO (cKO) system can achieve partial control over the timing and distribution of receptor inactivation using a specific promoter to direct Cre recombinase expression postnatally and/or in a selected cell subtype [e.g. *CaMK-IIα* driven-*cre* to study the effects of the adenosine A_{2A} receptor ($A_{2A}R$) in postnatal forebrain].

To achieve an even higher degree of precision in eliminating the $A_{2A}R$ from discrete regions on one side of the brain, and in pursuit of a better understanding of $A_{2A}R$ involvement in neurodegeneration, we adopted an AAV-Cre/*loxP* system. Adeno-associated virus (AAV)-Cre vectors were stereotactically infused (with an injection volume of 1 μl) into the left striatum of homozygous floxed $A_{2A}R$ adult mice, resulting in a cKO of the $A_{2A}R$ at the site and time of infection. The titer-dependence and time-course of neuronal Cre expression and $A_{2A}R$ loss, were characterized and visualized by immunohistochemistry. Cre expression was detectable 8 days post-infusion of AAV1 serotype vectors but the loss of $A_{2A}R$ was not evident until the 16th day, reaching a maximum extent at the 32nd day post-injection. The extent of the infected area depended on the titer of the infused vectors, estimated at 20% of calculated volume of the striatum using a low titer of 10^{12} gc/ml and reaching 80% when a higher titer of 10^{14} gc/ml was infused. In this model, the AAV-Cre/*loxP* cKO system provides a temporally and spatially controlled method to precisely eliminate $A_{2A}R$ in discrete brain regions providing additional advantages in exploring the neurobiology of adenosine receptors and their pathophysiology in models of CNS disease.

Disclosures: **A. Pisanu** , None; **M. Sena-Esteves**, None; **M.A. Schwarzschild**, None.

Support: DoD W81XWH-04-1-0881